Urinary vitamin D-binding protein levels are increased in patients with endometriosis

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Urinary vitamin D-binding protein levels are increased in patients with endometriosis

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SiHyun

<TABLE OF CONTENTS>

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

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Endometriosis, defined as the proliferation of endometrial tissue outside the uterine cavity, is one of the most common benign gynecologic disorders. However, diagnosis of endometriosis can be often confusing since no definite diagnostic biomarker is available, yet. Therefore, at present, direct visualization of the lesion and histologic confirmation through surgical procedures are essential for the definitive diagnosis of endometriosis. The purpose of this study was to investigate the clinical value of proteins secreted in urine in the diagnosis of endometriosis using proteomic analysis. Patients included in the study all underwent laparoscopy for different indications including pelvic masses, pelvic pain, suspicious endometriosis, infertility, and diagnostic evaluation, and only after pathologic confirmation were patients assigned to the endometriosis group. Urine samples were collected into sterile plastic tubes when the bladder was catheterized after induction of anesthesia. 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). Among 22 protein spots with significantly different expressions in urine of patients with and without endometriosis, vitamin D-binding protein (VDBP) was identified. Western blot analysis showed that the relative density of urinary VDBP was significantly higher in patients with endometriosis than the controls. Quantification of urinary vitamin D-binding protein corrected for creatinine (VDBP-Cr, nanogram per milligram) using ELISA revealed that urinary VDBP-Cr was significantly higher in patients with endometriosis than those without (111.96 ± 74.59 ng/mg Cr vs. 69.90 ± 43.76 ng/mg Cr, *P*=0.001). When urinary VDBP-Cr was combined with serum CA-125 levels, the diagnostic performance of detecting endometriosis showed sensitivity and specificity of 73.2% and 97.3% with cutoff value of 2755.71. Urinary vitamin D-binding protein levels are increased in patients with endometriosis and may be used as a simple and easily obtained diagnostic biomarker for endometriosis.

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

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I. INTRODUCTION

Endometriosis, defined as the proliferation of endometrial tissue outside the uterine cavity, is one of the most common benign gynecologic disorders. This disease is present in about 10% of all reproductive-aged women, and its prevalence increases to 20%-50% in infertile women [1]. However,

diagnosis of endometriosis can be often confusing since no definite diagnostic biomarker is available, yet. Imaging techniques, such as ultrasound and magnetic resonance imaging, also have been shown to be unreliable in the diagnosis or staging of this disease. Therefore, at present, direct visualization of the lesion and histologic confirmation through surgical procedures are essential for the definitive diagnosis of endometriosis. Although laparoscopy is a minimally invasive procedure, it requires general anesthesia and surgical skills, and it involves potential complications as well as procedural costs.

Despite numerous investigative efforts and recent advances in understanding of the molecular basis of endometriosis, substantial gap remains in the development of effective noninvasive diagnostic biomarker for endometriosis. Therefore, newly available technologies could play a key role in the identification of early, new, novel biological markers for endometriosis. Recently, several authors 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 than those without [2-6]. Proteomic approaches allow the comparison of protein expression in cells, tissues or body fluids under different conditions, and the study of protein function and protein–protein interactions may provide more information about

the disease than the application of genomics tools since low correlation exists between the genotype and phenotype in endometriosis[7].

Urine may be an ideal candidate as a source for biomarkers because it can be easily obtained in relatively large quantities using non-invasive procedures. Clinical importance of urinary angiogenic factors and cytokines has been demonstrated not only in urogenial diseases, but also in other systemic diseases including heart failure and brain tumor [8-13]. Recent studies indicate that proteomic analysis of urine may lead to new potential biomarkers for various systemic disease such as ovarian cancer and coronary artery disease [14-15]. We have previously demonstrated the significance of urinary angiogenic factors in patients with endometriosis[12]. Therefore, we hypothesized that certain proteins might be excreted in urine in women with endometriosis compared with those without. The purpose of this study is to perform proteomic analysis of urine of the patients with and without endometriosis and to identify potential urinary novel biomarkers for endometriosis.

II. MATERIALS AND METHODS

1. Study population & Sample Collection

Ninety-five patients aged 20 to 47 years participated in this study after giving written informed consent. The study was approved by the Institutional Review Board of Gangnam Severance Hospital. Patients included in the study all underwent laparoscopy for different indications including pelvic masses, pelvic pain, suspicious endometriosis, infertility, and diagnostic evaluation. Pre-treatment serum CA-125 levels were measured in all patients, using CA-125 II ECLIA (electrochemiluminescence immunoassay) on the Roche/Hitachi Modular Analytics E170 (Roche Diagnostics, Tokyo, Japan). At the time of surgery, all possible endometriotic lesions were excised and sent to pathology for confirmation. Only after pathologic confirmation were patients assigned to the endometriosis group. The extent of endometriosis was determined using the American Society of Reproductive Medicine (ASRM) revised classification [16]. Fifty-seven patients had histologically confirmed endometriosis: 5 patients were classified as minimal-to-mild disease (Stages I & II) and 52 patients as moderate-to-severe disease (Stages III and IV). Thirty-eight patients participated as controls. The controls included 18 cases of dermoid cyst and 20 cases of benign ovarian cysts including serous cystadenoma and paratubal cyst. Post-menopausal women, previous hormone or GnRH agonist users and patients who had adenomyosis, endometrial cancer,

endometrial hyperplasia or endometrial polyps, infectious diseases, chronic or acute inflammatory diseases, malignancy, autoimmune diseases, or cardiovascular diseases were excluded. 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 time of the menstrual cycle was recorded for each patient: proliferative phase, beginning of menses until 14 days before the next menses, and secretory phase, 1-13 days before the next menses.

Urine samples were collected from 57 women with histologically proven endometriosis and 38 women without histological or laparoscopic evidence of endometriosis. The urine (20 ml each) was collected into sterile plastic tubes when the bladder was catheterized after induction of anesthesia and added 1 x protease inhibitor cocktail (Roche, Germany). Urine samples were immediately centrifuged at 1000 X *g* for 10 min and sediment- free urine samples were obtained. Urine aliquots were frozen at -80°C until further analysis. Since previous studies have demonstrated that the collection of 24-h and first-morning urine is less practical for proteomic analysis and may have a problem of contamination of proteins from overgrown bacteria or bladder epithelial cells, no effort was made to obtain samples at a specific time of the day [17-18].

2. Proteomics

Each urine samples (20 ml) were concentrated to 0.5 ml using Vivaspin (2,000 MW cut-off, Sartorius, Hannover, Germany) and proteins in the concentrated urine was 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 2D buffer (7 M urea, 2 M thiourea, 0.4% (w/v) DTT, and 4% (w/v) CHAPS) and centrifuged at 13,000 x g for 15 min. The concentrations of proteins in the supernatants were determined by Bradford method.

For 1st dimensional electrophoresis, 450μg of each urine proteins were rehydrated overnight at room temperature onto linear pH4-7 (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 (TBP) and 15 mM acrylamide, respectively, for 15 min at ambient temperature.

The 2-D separations were performed on 8– 16% (v/v) linear gradient SDS-polyacrylamide gels. After protein separations, the 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, South Korea) overnight. The gels were destained in 1% (v/v) acetic acid for 4h 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 were analyzed between women with and without endometriosis using ImageMaster V software (GE Healthsciences).

3. Identification of proteins 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 added with 10 ul of Trypsin solution (12 ng/ml, Promega, Madison, USA). Following discarding excess trypsin and adding 20 ul of 50 mM ammonium bicarbonate, the gels were incubated overnight at 37 ℃. The tryptic digest solutions were concentrated and cleaned up using a C18 micro column (Millipore, Billerica, MA) as instructed by manufacture.

The resulting tryptic peptides were separated and analyzed using reversed phase capillary HPLC directly coupled to a Finnigan LCQ ion trap mass spectrometer (LC-MS/MS) [19]. Both of a 0.1 x 20 mm trapping and a 0.075 x 130mm resolving column were packed with Vydac 218MS low trifluoroacetic acid C18 beads (5㎛ in size, 300Å in pore size; Vydac, Hesperia, CA, USA) and placed in-line. Following the peptides were bound to the trapping column for 10 min at with 5% (v/v) aqueous acetonitrile containing 0.1% (v/v) formic acid, then 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㎕/min. For tandem mass spectrometry, each scan cycle included a full mass scan range mode (m/z 300– 2000Da) and up to three product ion scans with relative collision energy of 55%. The individual spectra from MS/MS were processed using the Bioworks 3.3 software (Thermo Fisher, San Jose, CA). The generated peak list files were used to query NCBI human database using the MASCOT program (http://www.matrixscience.com). Variable modifications of methionine (oxidation) and cysteine (propionamide), peptide mass tolerance at 2 Da, MS/MS ion mass tolerance at 1Da, allowance of missed cleavage at 1, and charge states (+1, +2, and +3) were search parameters. Matches with peptide(s) with only significant hits as defined by MASCOT probability analysis were accepted.

4. Western Blot

Urines from 8 samples (4 endometriosis and 4 control patients), different from those subjected to 2DE analyses, were quantified using a BCA assay (Pierce). For western blot analyses, equal amounts of urine proteins (70 ㎍) were run onto 4-20% gradient polyacrylamide gel (KomaBiotech, Seoul, Korea) and transferred to PVDF membranes for 50 min at 15 V using a EZway blotting system (KomaBiotech). Blots were blocked for 1 hr 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 3 times washes with TBS-T, the blots were incubated at 4℃ overnight with vitamin D binding protein

(VDBP) (Santa curz biotechonlogy ,sc-69771) antibody at a concentration of 1:700 in TBS-T. The membranes were washed with TBS-T solution and incubated with horseradish peroxidase -conjugated goat anti-mouse IgG (invitrogen) at a 1:2,000 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. England). The relative density of the protein bands was quantified by Gel Doc XR+ (Bio-Rad)

5. Enzyme-linked immunosorbent assay (ELISA)

Concentrations of VDBP in urine were measured (100$\mu\ell$, diluted 1:2) using specific commercial sandwich enzyme-linked immunosorbent assays (ELISA) according to the manufacturer's protocols (ALPCO Diagnostics; Salem, NH; enzyme immunoassay no. 30-2314) and expressed as ng/ml. The minimum detectable concentrations 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 milligrams).

6. Statistical Analysis

Data were expressed as mean ± SD or median (interquartile ranges)

where appropriate. A Student's t-test was used to compare the clinical and laboratory characteristics of the patients with endometriosis and control group. For the differences of VDBP-Cr through the menstrual cycles, the Kruskal–Wallis test followed by the Dunn procedure was used. The levels of significance in correlation were calculated by Pearson's correlation coefficient or Spearman rank correlation coefficient, where appropriate. The diagnostic performance of urinary VDBP-Cr was assessed using receiver operating characteristic (ROC) curves [20]. An ROC curve is a plot of the sensitivity of a test versus its false-positive rate, in other words, 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) and the area under the ROC curve was calculated as a measure of the ability of each potential marker to discriminate between endometriosis cases and controls. An AUC of 0.5 indicates classifications assigned by chance. Based on ROC analysis, the best statistical cut-off value of VDBP-Cr (the point at which the sum of false-positives and false-negatives is less than any other point) was calculated. Sensitivity and specificity for selected cut-off points were then assessed. SPSS 16.0 package (SPSS Inc, Chicago, Il.) was used for statistical analysis and statistical significance was accepted at $p<0.05$.

III. RESULTS

1. Clinical characteristics

The clinical characteristics of patients are shown in Table 1. The mean age (mean ± S.D.) of endometriosis patients and controls was 34.22 ± 6.88 years and 32.76 ± 10.26 years, respectively. There was no significant differences in the mean gravidarum, mean parity, and mean BMI between the two groups, while serum CA-125 levels were significantly higher in endometriosis patients than in controls (83.09 ± 120.93 IU/mL vs. 20.57 ± 13.25 IU/mL, respectively, $P < 0.001$). No significant difference was noted in mean urine creatinine levels between the two groups.

Table 1. Clinical Characteristics of Study Group

	Endometriosis (N=57)	Control (N=38)	P value
Age(years)	34.22 ± 6.88	32.76 ± 10.26	0.443
Gravidarum	1.02 ± 1.31	1.24 ± 1.46	0.448
Parity	0.60 ± 0.86	0.58 ± 0.86	0.923
BMI(㎡/kg)	18.10 ± 7.30	17.72 ± 7.76	0.809
CA-125(IU/ml)	83.09 ± 120.93	20.57 ± 13.25	<0.001
Urine Creatinine (mg/dl)	103.15 ± 52.36	88.39 ± 68.83	0.239

Data are expressed as mean ± S.D.

2. Proteomics

Well resolved protein profiles were obtained when urine protein samples were analyzed by the two-dimensional electrophoresis. Five hundred twelve protein spots in the pH range 4-7 were observed by two-dimensional (2D) gel image analyses. Some variations in protein separation and spot intensity on 2D gels appeared between women with endometriosis group and the controls. Image analyses of 2D gels revealed that 22 protein spots in their intensities (%v) on the sample group are apparently much higher differences than those on the control group (Figure 1). Of those 22 upregulated 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. Among those 5 spots, one spot was identified to be vitamin D binding protein (*pI* 5.40, Mr 52.93) by LC-MS/MS. We decided that vitamin D binding protein 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 vs. 0.0006 ± 0.001, respectively, $P<0.001$).

3. Biomarker Validation

Western blot analysis of 8 urine samples (4 samples from endometriosis patients, 4 samples from the controls) probed with VDBP antibody showed that all samples showed were positive for VDBP (Figure 2). Quantification of the relative density of the protein bands indicated that the relative density of urinary VDBP was significantly higher in patients with endometriosis than the controls (2.48±0.11 vs. 1.15±0.26, $P<0.001$) (Figure 2).

Quantification of urinary VDBP using ELISA in 57 patients with endometriosis and 38 patients without endometriosis showed that urinary VDBP levels are significantly increased in women with endometriosis than those without (106.12 ± 74.55 ng/ml vs. 58.68 ± 60.82 ng/ml, respectively, $P=0.001$) (Figure 3). When urinary VDBP levels were corrected for creatinine (vitamin D binding protein to creatinine ratio, VDBP-Cr), VDBP-Cr was also higher in women with endometriosis than those with statistically significance (111.96 ± 74.59 ng/mg Cr vs. 69.90 ± 43.76 ng/mg Cr, respectively, $P=0.001$) (Figure 3).

Figure 4 shows the cyclic variation of VDBP-Cr in patients with endometriosis and controls (data are expressed in median and interquartile range[IQR]). When VDBP-Cr was evaluated according to the menstrual cycle, no significant difference in the median VDBP-Cr was noted during the proliferative phase between the two groups. The median VDBP-Cr in patients with and without endometriosis during the secretory phase were 91.20(IQR 53.31-143.32) and 54.13(IQR 36.02-82.14), respectively, and the difference was

statistically significant (*P*=0.042). We also evaluated the correlations between VBDP-Cr and other clinical features. However, no significant correlations were noted between VDBP-Cr and the severity of the disease nor serum CA-125 levels (data not shown).

To assess the diagnostic utility of VDBP-Cr in diagnosing endometriosis, we examined the receiver operating characteristics (ROC) curve of VDBP-Cr and serum CA-125 levels (Figure 5). The diagnostic performance of CA-125 alone was assessed at a cut-off point of 35 IU/mL, which is the most generally reported in the literature. The AUC for CA-125 was 0.857 (95% confidence interval [CI]: 0.781-0.933) with sensitivity of 69.6% and specificity of 91.9%. For VDBP-Cr, the AUC was 0.678 (95% CI: 0.569-0.787) and the cut-off value was 87.83 with 57.9% sensitivity and 76.3% specificity. We also evaluated the diagnostic power of the combined marker, which was obtained by multiplying VDBP-Cr by serum CA-125 levels, and ROC curve was constructed (Figure 5). The AUC for the combined marker was 0.874 (95% CI: 0.802-0.945) and the optimal cut-off value was 2755.71 with sensitivity of 73.2% and specificity of 97.3%.

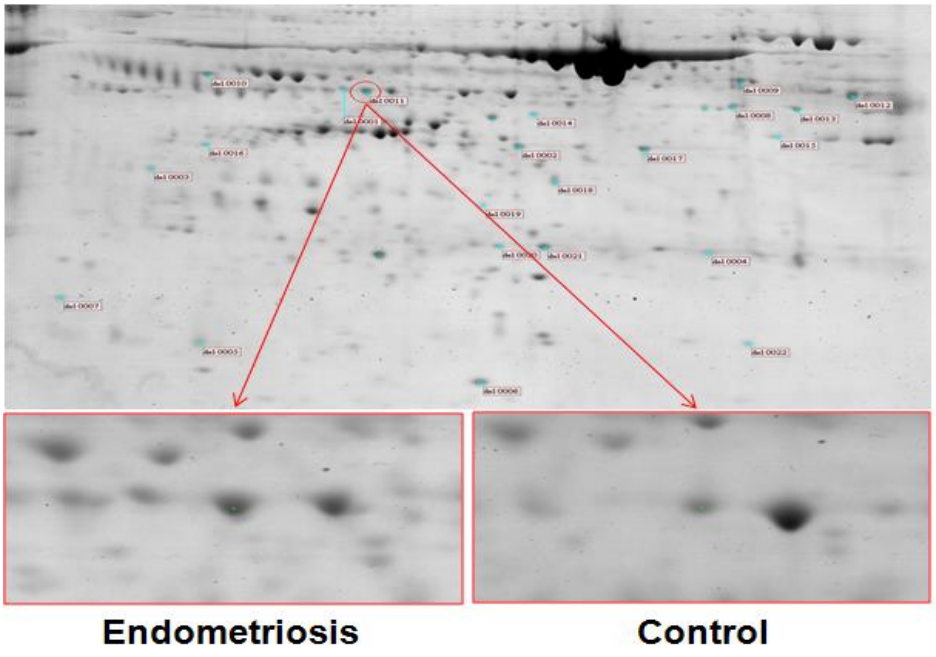

Figure 1. Proteomic analysis of highly expressed proteins in the urine of women with endometriosis. Twenty-two spots with significantly different expression (% volume) in urine of women with and without endometriosis were identified. The protein spot with circled in red indicates vitamin D binding protein.

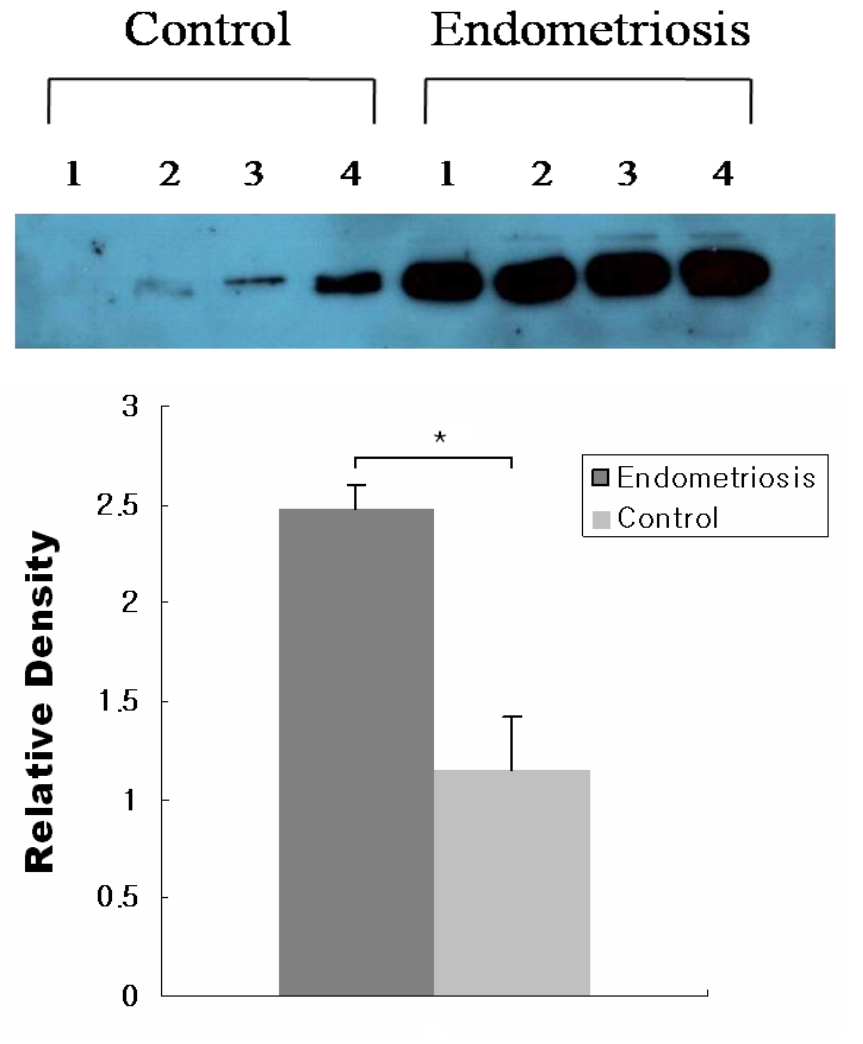

Figure 2. Western blot analysis of 8 urine samples probed with vitamin D binding protein (VDBP) antibody. The expression of VDBP was significantly higher in patients with endometriosis than the controls. Data are expressed as mean±S.D.

*P<0.001

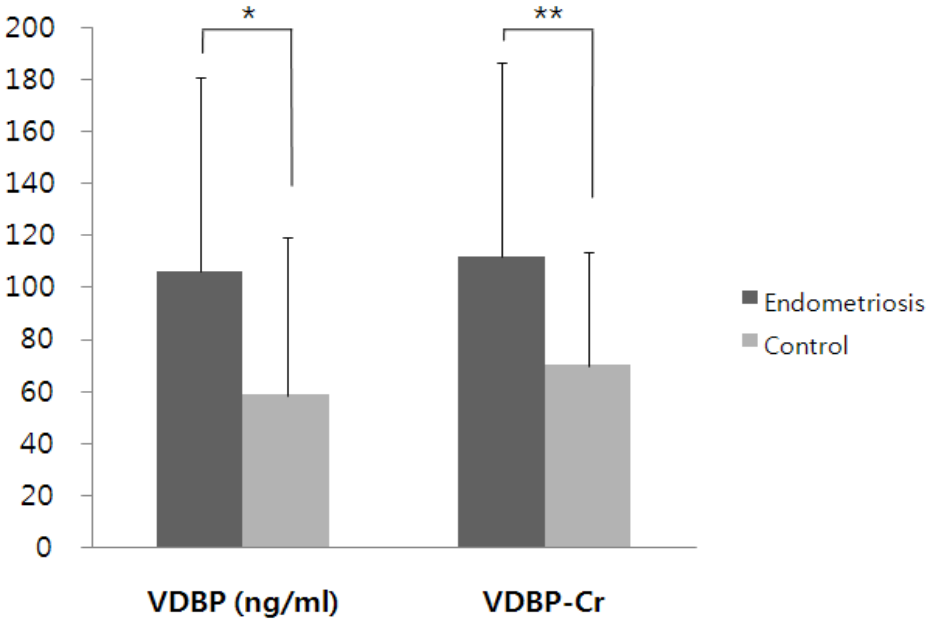

Figure 3. Urinary Vitamin D binding protein (VDBP) levels and Vitamin D binding protein-to-creatinine ratio (VDBP-Cr) in patients with endometriosis and controls. Data are expressed as mean±S.D.

*P=0.001 **P=0.001

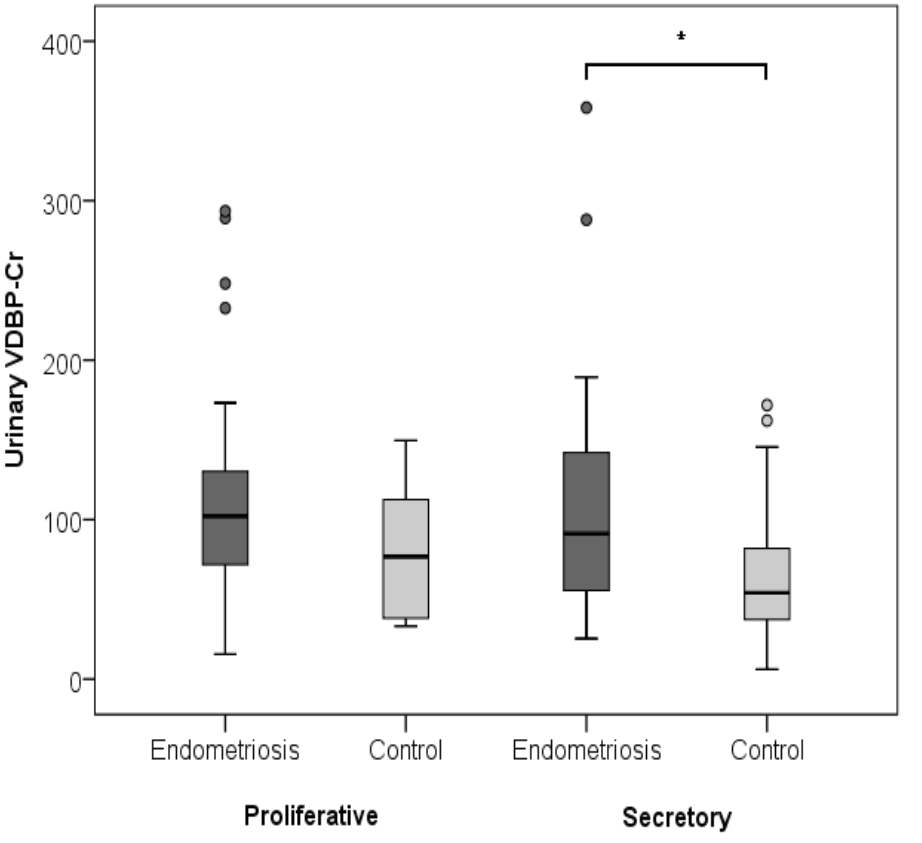

Figure 4. Urinary vitamin D binding protein-to-creatinine ratio (VDBP-Cr) in patients with endometriosis and controls according to the menstrual cycle. Urinary VDBP-Cr levels were significantly higher in patients with endometriosis than controls during secretory phase of the menstrual cycle (Kruskal-Wallis test followed by Dunn procedure). Data are expressed as median(interquartile range).

* *P*=0.042

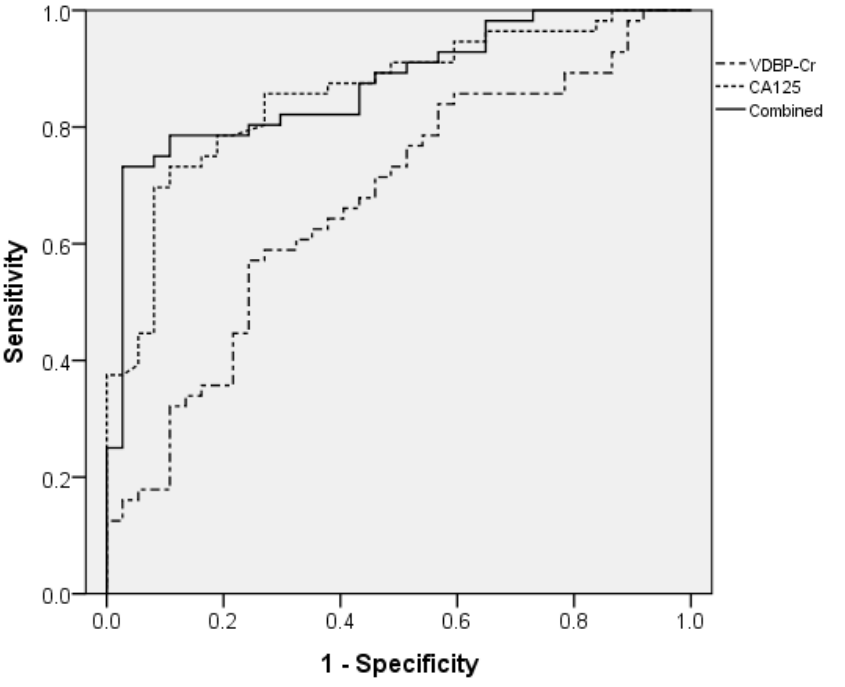

Figure 5. Receiver operating characteristic curves of serum CA-125, urinary vitamin D binding protein-to-creatinine (VDBP-Cr) and the combined marker (serum CA-125 levels multiplied by vitamin D binding protein-to-creatinine (VDBP-Cr) for the differential diagnosis between patients with endometriosis and controls.

IV. DISCUSSION

In the present study, we identified, using proteomic techniques, the presence of up-regulated vitamin D binding protein in urine of women with endometriosis compared with those without. We further validated the presence of this urinary protein using western blot and ELISA analysis, and it showed that urinary vitamin D binding protein is significantly elevated in women with endometriosis. The diagnostic power of urinary vitamin D binding protein normalized to urine creatinine concentrations yielded sensitivity and specificity of 57.9% and 76.3%, respectively, at cut-off value of 87.83. When combined with serum CA-125 levels, it demonstrated fairly high sensitivity and specificity in detecting endometriosis with the overall sensitivity and specificity of 73.2% and 97.3%, respectively.

Urine may be considered as an ideal body fluid as a potential candidate for biomarker. Compared with other body fluids, urine has several characteristics that make it a preferred choice for biomarker discovery in proteomic approach. These advantages include easily obtainable in large quantities using simple non-invasive procedures, solubility of urinary peptides and lower molecular mass proteins, and relative stability of the urinary protein content, which is in contrast to blood for which activation of proteases is inevitably associated with its collection [21]. Therefore, recently, a number of papers were published concerning the proteomic analysis of urine for the search of novel biomarkers for several different diseases [14-15]. Zimmerli et al.

developed the coronary artery disease-specific panel using urinary proteomics techniques which showed sensitivity and specificity of 98% and 83%, respectively, for the prediction of coronary artery disease and Ye et al. reported proteomic-based discovery and characterization of urinary glycosylated eosinophil-derived neurotoxin and COOH-terminal osteopontin fragments for ovarian cancer [14-15]. More recently, urinary proteomic techniques have been applied to the biomarker discovery of 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[2].

In this study, we identified that urinary VDBP is increased in patients with endometriosis. VDBP, known as group-specific component or Gc-globubin, is the major plasma carrier protein of vitamin D metabolites, being 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 [22]. However, less than 5% of circulating DBP is actually complexed with vitamin D metabolites, leaving a considerable amount of the protein available for other functions that are closely related to the pathogenesis of endometriosis. This protein seems to be involved in transportation of fatty and endotoxins, and is known to be an important factor in the actin scavenging system [23-25]. In addition, there is a growing body of evidence suggesting that VDBP plays an important role in immune system by acting as a chemotactic factor in the recruitment of neutrophil neutrophils, monocytes, and fibroblasts

[26-28] Also, at sites of inflammation, consecutive contact of VDBP with B and T cells is able to convert VDBP into a potent macrophage activating factor.[29] The fact that the concentration of the Gc-globulin is influenced by other hormones such as estrogen and interleukin-1, which are known to be involved with endometriosis, also adds to the biological plausibility of connecting this protein with the pathogenesis of endometriosis.[30-31]

Despite the diverse and important roles of VDBP, there are only limited number of studies evaluated the expression of this molecule in endometriosis. One study showed no significant differences in serum and peritoneal fluids VDBP concentrations between women with and without endometriosis, while the other using high-resolution two-dimensional gel electrophoresis reported that one isoform of VDBP was significantly lower in the peritoneal fluid of women with endometriosis compared with controls and the expression was further decreased with the treatment of gonadotropin-releasing hormone agonist [32-34] More recently, proteomic analysis of serum using two-dimensional difference gel electrophoresis showed that the abundance of vitamin D-binding protein was higher in endometriosis patients and suggested that the inability to sufficiently activate macrophages' phagocytotic function in those carrying the GC*2 polymorphism may be associated with the implantation of endometriotic tissues to the peritoneal cavity [35].

Evaluation of urinary VDBP has been reported previously and

indicated that excessive urinary VDBP are associated with long-term cadium exposure and type I diabetes [36-37]. Our results showed that urinary VDBP is increased in patients with endometriosis. When urinary VDBP was evaluated according to the menstrual cycle, the levels were significantly higher in patients with endometriosis than controls during secretory phase of the menstrual cycle. However, we found no significant cyclic variations of urinary VDBP in both endometriosis group and control group. Although there are no previous studies indicating the cyclic variations of urinary VBDP, our findings are in line with results showing that plasma VDBP expressions are not related to the phase of the menstrual cycle [33, 38].

One potential limitation of this study concerns 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, and although we only included the patients surgically proven to be free of endometriosis, majority of our control group had various other benign diseases, which may have different effects on the levels of urinary VDBP.

V. CONCLUSION

In conclusion, this is the first to report the identification and validation of a urinary biomarker, urinary VDBP, with the diagnostic accuracy of detecting endometriosis. Although the diagnostic accuracy of urinary VDBP did not exceed that of serum CA-125 in this study, the combination of these two markers showed relatively high sensitivity and specificity and it seems clear that measurement of the urinary biomarker could be an extremely valuable for the reliable diagnosis of endometriosis. Since only small number of patients included in study was minimal-to-mild disease, it is difficult to determine the diagnostic accuracy of this combined marker for minimal-to-mild disease. Also, although the expressions of this protein have been found in the uterus, serum and the peritoneal fluids, it is unclear why or how VDBP is excreted in this specific way in urine [32-34, 39] Therefore, further research with large number of patients is needed to investigate the pathophysiology and possible role of urinary VDBP in patients with endometriosis.

REFERENCES

1. Taylor RN, Lebovic DI, Mueller MD. Angiogenic factors in endometriosis. Ann N Y Acad Sci 2002;955:89-100; discussion 18, 396-406.
2. Tokushige N, Markham R, Crossett B, Ahn SB, Nelaturi VL, Khan A, et al. Discovery of a novel biomarker in the urine in women with endometriosis. Fertil Steril 2011;95(1):46-9.
3. Seeber B, Sammel MD, Fan X, Gerton GL, Shaunik A, Chittams J, et al. Proteomic analysis of serum yields six candidate proteins that are differentially regulated in a subset of women with endometriosis. Fertil Steril 2010;93(7):2137-44.
4. Liu H, Lang J, Zhou Q, Shan D, Li Q. Detection of endometriosis with the use of plasma protein profiling by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry. Fertil Steril 2007;87(4):988-90.
5. Ferrero S, Gillott DJ, Remorgida V, Anserini P, Leung KY, Ragni N, et al. Proteomic analysis of peritoneal fluid in women with endometriosis. J Proteome Res 2007;6(9):3402-11.
6. Ametzazurra A, Matorras R, Garcia-Velasco JA, Prieto B, Simon L, Martinez A, et al. Endometrial fluid is a specific and non-invasive biological sample for protein biomarker identification in endometriosis. Hum Reprod 2009;24(4):954-65.
7. Poliness AE, Healey MG, Brennecke SP, Moses EK. Proteomic approaches in endometriosis research. Proteomics 2004;4(7):1897-902.
8. Dvorak HF. Vascular permeability factor/vascular endothelial growth factor: a critical cytokine in tumor angiogenesis and a potential target for diagnosis and therapy. J Clin Oncol 2002;20(21):4368-80.
9. Matsumoto K, Kanmatsuse K. Elevated vascular endothelial growth factor levels in the urine of patients with minimal-change nephrotic syndrome. Clin Nephrol 2001;55(4):269-74.
10. Buhimschi CS, Norwitz ER, Funai E, Richman S, Guller S, Lockwood CJ, et al. Urinary angiogenic factors cluster hypertensive disorders and identify women with severe preeclampsia. Am J Obstet Gynecol 2005;192(3):734-41.
11. Rivera M, Talens-Visconti R, Sirera R, Bertomeu V, Salvador A, Cortes R, et al. Soluble TNF-alpha and interleukin-6 receptors in the urine of heart failure patients. Their clinical value and relationship with plasma levels. Eur J Heart Fail 2004;6(7):877-82.
12. Cho SH, Oh YJ, Nam A, Kim HY, Park JH, Kim JH, et al. Evaluation of serum and urinary angiogenic factors in patients with endometriosis. Am J Reprod Immunol 2007;58(6):497-504.

13. Smith ER, Zurakowski D, Saad A, Scott RM, Moses MA. Urinary biomarkers predict brain tumor presence and response to therapy. Clin Cancer Res 2008;14(8):2378-86.
14. Ye B, Skates S, Mok SC, Horick NK, Rosenberg HF, Vitonis A, et al. Proteomic-based discovery and characterization of glycosylated eosinophil-derived neurotoxin and COOH-terminal osteopontin fragments for ovarian cancer in urine. Clin Cancer Res 2006;12(2):432-41.
15. Zimmerli LU, Schiffer E, Zurbig P, Good DM, Kellmann M, Mouls L, et al. Urinary proteomic biomarkers in coronary artery disease. Mol Cell Proteomics 2008;7(2):290-8.
16. Revised American Society for Reproductive Medicine classification of endometriosis: 1996. Fertil Steril 1997;67(5):817-21.
17. Bottini PV, Ribeiro Alves MA, Garlipp CR. Electrophoretic pattern of concentrated urine: comparison between 24-hour collection and random samples. Am J Kidney Dis 2002;39(1):E2.
18. Hoorn EJ, Pisitkun T, Zietse R, Gross P, Frokiaer J, Wang NS, et al. Prospects for urinary proteomics: exosomes as a source of urinary biomarkers. Nephrology (Carlton) 2005;10(3):283-90.
19. Bahk YY, Kim SA, Kim JS, Euh HJ, Bai GH, Cho SN, et al. Antigens secreted from Mycobacterium tuberculosis: identification by proteomics approach and test for diagnostic marker. Proteomics 2004;4(11):3299-307.
20. Hanley JA, McNeil BJ. The meaning and use of the area under a receiver operating characteristic (ROC) curve. Radiology 1982;143(1):29-36.
21. Decramer S, Gonzalez de Peredo A, Breuil B, Mischak H, Monsarrat B, Bascands JL, et al. Urine in clinical proteomics. Mol Cell Proteomics 2008;7(10):1850-62.
22. Daiger SP, Schanfield MS, Cavalli-Sforza LL. Group-specific component (Gc) proteins bind vitamin D and 25-hydroxyvitamin D. Proc Natl Acad Sci U S A 1975;72(6):2076-80.
23. Berger D, Beger HG. Evidence for endotoxin binding capacity of human Gc-globulin and transferrin. Clin Chim Acta 1987;163(3):289-99.
24. Williams MH, Van Alstyne EL, Galbraith RM. Evidence of a novel association of unsaturated fatty acids with Gc (vitamin D-binding protein). Biochem Biophys Res Commun 1988;153(3):1019-24.
25. Lee WM, Galbraith RM. The extracellular actin-scavenger system and actin toxicity. N Engl J Med 1992;326(20):1335-41.
26. Perez HD, Kelly E, Chenoweth D, Elfman F. Identification of the C5a des Arg cochemotaxin. Homology with vitamin D-binding protein (group-specific component globulin). J Clin Invest 1988;82(1):360-3.
27. Kew RR, Webster RO. Gc-globulin (vitamin D-binding protein)

enhances the neutrophil chemotactic activity of C5a and C5a des Arg. J Clin Invest 1988;82(1):364-9.
28. Piquette CA, Robinson-Hill R, Webster RO. Human monocyte chemotaxis to complement-derived chemotaxins is enhanced by Gc-globulin. J Leukoc Biol 1994;55(3):349-54.
29. Yamamoto N, Naraparaju VR. Role of vitamin D3-binding protein in activation of mouse macrophages. J Immunol 1996;157(4):1744-9.
30. Rejnmark L, Lauridsen AL, Vestergaard P, Heickendorff L, Andreasen F, Mosekilde L. Diurnal rhythm of plasma 1,25-dihydroxyvitamin D and vitamin D-binding protein in postmenopausal women: relationship to plasma parathyroid hormone and calcium and phosphate metabolism. Eur J Endocrinol 2002;146(5):635-42.
31. Guha C, Osawa M, Werner PA, Galbraith RM, Paddock GV. Regulation of human Gc (vitamin D--binding) protein levels: hormonal and cytokine control of gene expression in vitro. Hepatology 1995;21(6):1675-81.
32. Borkowski J, Gmyrek GB, Madej JP, Nowacki W, Goluda M, Gabrys M, et al. Serum and peritoneal evaluation of vitamin D-binding protein in women with endometriosis. Postepy Hig Med Dosw (Online) 2008;62:103-9.
33. Ferrero S, Gillott DJ, Anserini P, Remorgida V, Price KM, Ragni N, et al. Vitamin D binding protein in endometriosis. J Soc Gynecol Investig 2005;12(4):272-7.
34. Ferrero S, Gillott DJ, Remorgida V, Anserini P, Ragni N, Grudzinskas JG. GnRH analogue remarkably down-regulates inflammatory proteins in peritoneal fluid proteome of women with endometriosis. J Reprod Med 2009;54(4):223-31.
35. Faserl K, Golderer G, Kremser L, Lindner H, Sarg B, Wildt L, et al. Polymorphism in vitamin D-binding protein as a genetic risk factor in the pathogenesis of endometriosis. J Clin Endocrinol Metab 2011;96(1):E233-41.
36. Thrailkill KM, Jo CH, Cockrell GE, Moreau CS, Fowlkes JL. Enhanced excretion of vitamin D binding protein in type 1 diabetes: a role in vitamin D deficiency? J Clin Endocrinol Metab 2011;96(1):142-9.
37. Uchida M, Teranishi H, Aoshima K, Katoh T, Kasuya M, Inadera H. Elevated urinary levels of vitamin D-binding protein in the inhabitants of a cadmium polluted area, Jinzu River basin, Japan. Tohoku J Exp Med 2007;211(3):269-74.
38. Nielsen HK, Brixen K, Bouillon R, Mosekilde L. Changes in biochemical markers of osteoblastic activity during the menstrual cycle. J Clin Endocrinol Metab 1990;70(5):1431-7.
39. Cooke NE, McLeod JF, Wang XK, Ray K. Vitamin D binding protein:

genomic structure, functional domains, and mRNA expression in tissues. J Steroid Biochem Mol Biol 1991;40(4-6):787-93.

ABSTRACT(IN KOREAN)

자궁내막증 환자의 소변에서 증가된 vitamin D binding protein의 유용성

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조 시 현

연구목적: 자궁내막증은 자궁내막조직이 자궁내막이 자궁내의 정상 위치 밖에 존재하는 것으로 만성골반통 및 불임과 연관이 있는 비교적 흔한 부인과적 질환 중 하나이다. 그러나 아직까지 자궁내막증을 진단할 수 있는 효과적인 생표지자가 개발되지 않고 있으며 현재까지 자궁내막증의 진단은 수술 후 조직학적 진단에 의존하고 있다. 따라서 본 연구의 목적은 자궁내막증 환자의 소변에 대하여 단백체기법을 적용하여 자궁내막증 환자의 소변에서 발견되는 단백질을 규명하고, 자궁내막증의 진단적 생표지자로서의 임상적 유용성에 대해 분석해보고자 하였다.

연구방법: 골반종괴, 골반통, 불임, 또는 자궁내막증이 의심되어 복강경 수술을 시행받은 환자군을 대상으로 연구를 진행하였다. 수술 후 조직학적으로 자궁내막증으로 진단받은 경우에만 자궁내막증 군으로 분류하였다. 소변은 전신 마취 후 도뇨관을 삽입할 때 sterile plastic tube에 채취하였다. 소변에 대해 proteomic techniques and mass spectometry를 시행하여 자궁내막증 환자군과 대조군 환자의 소변에서 발견되는 단백질에 대해 Western blot과 sandwich enzyme-linked immunosorbent assays (ELISA)를 이용하여 정량하였다.

결과: Proteomic techniques and mass spectometry를 통하여 자궁내막증 환자와 대조군의 소변에서 22개의 단백질 반점이 통계학적으로 유의한 차이를 보이는 것으로 나타났고 이 중 하나가 vitamin D-binding protein(VDBP)으로 규명되었다. Western blot 결과 자궁내막증 환자의 소변에서 vitamin D-binding protein의 상대 밀도가 대조군에 비해 의미있게 높은 것으로 나타났다. 소변에 대한 ELISA 및 소변 크레아티닌 수치 보정 (VDBP-Cr, nanogram per milligram) 결과 자궁내막증 환자의 소변 VDBP-Cr이 대조군에 비해 통계학적으로 유의하게 증가되어 있었다 (111.96 ± 74.59 ng/mg Cr vs. 69.90 ± 43.76 ng/mg Cr, P=0.001). 소변 VDBP-Cr과 혈청 CA-125 수치를 같이 사용할 경우 자궁내막증을 진단할 수 있는 진단적 가치는 기준수치가 2755.71에서 민감도와 특이도가 각각 73.2%와 97.3%로 나타났다.

결론: 자궁내막증 환자의 소변에서 VDBP이 증가되어 있으며 쉽고 간편하게 채취 가능한 소변이 자궁내막증의 진단에 사용될 수 있다는 증거를 제시하였다.

핵심되는 말 : 생표지자, 자궁내막증, 단백체기법, 소변, vitamin d-binding protein