Evaluation of osteopontin expression in the eutopic endometrium and plasma osteopontin levels in patients with endometriosis

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The Master's Thesis submitted to the Department of Medicine, the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Master of Medical Science

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To my parents, I would like to express thanks for bring me into this world and raising me to this day. Finally I give a vote of thanks to my wife and my children, valuable helpers of this work.

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Abstract

Evaluation of osteopontin expression in the eutopic endometrium and plasma osteopontin levels in patients with endometriosis

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Objectives: The aim of this study was to evaluate mRNA expression of osteopontin (OPN) in eutopic endometrium and plasma osteopontin levels in patients with endometriosis.

Materials and Methods: 64 patients with histologically confirmed endometriosis and 40 patients without endometriosis participated in this study. Endometrial biopsies were performed during exploration laparotomy or operative laparoscopy and blood samples were collected by peripheral venous puncture before anesthesia. Real time quantitative RT-PCR was performed to quantify OPN mRNA expression of endometrial tissues, and plasma concentrations of OPN were measured using specific commercial sandwich enzyme-linked immunosorbent assays (ELISA).

Results: The mean OPN mRNA expression (mean±S.E.) was significantly higher in endometrial tissues of women with endometriosis than in endometrial tissues of controls (783.95±256.45 vs. 83.37±34.47, P=0.029). The mean ± S.E. levels of plasma OPN in patients with endometriosis and the controls were 407.31±37.80 ng/ml and 160.07±18.94 ng/ml, respectively (P < 0.001). Plasma OPNhave strong positive correlation between serum CA-125 levels (r=0.375, p=0.001), but no correlations between plasma OPN and the severity of the disease was noted. ROC analysis for plasma OPN revealed an

AUC of 0.846, sensitivity of 93.6% and specificity of 65.7% at a cut-off value of 167.68 ng/ml.

Conclusions: Expressions of OPN in eutopic endometrium and plasma OPN levels are increased in the patients with endometriosis. OPN may be involved in the pathogenesis of endometriosis and further evaluation of plasma OPN as a potential biomarker for the diagnosis of endometriosis is warranted.

Key words: Endometriosis, endometrium, mRNA, osteopontin, plasma

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I. Introduction

Endometriosis is a chronic disease manifested by pelvic pain and infertility and defined as the presence of endometrial glands and stroma within the pelvic peritoneum and other extra-uterine sites. It is thought to affect up to 10% of women of reproductive age and the frequency rises to 50% in women with pelvic pain, infertility or both.(1)

Endometriosis is considered a polygenically inherited disease with a complex, multifactorial etiology. Susceptibility to endometriosis depends on the complex interaction of genetic, immunological, hormonal, and environment factors.(2) Sampson's theory of transplantation of endometrial tissue on the pelvic

peritoneum through retrograde menstruation is the most widely accepted explanation for the development of pelvic endometriosis.(3) However, because retrograde menstruation is observed in almost all cycling women, the much lower prevalence of endometriosis suggests that other factors must determine the susceptibility to developing endometriosis.(4) This process is determined by the quantity and quality of retrograde menstruation, the subclinical peritoneal fluid inflammation occurring during retrograde menstruation and local peritoneal factors such as tumor necrosis factor-α (TNF-α), matrix metalloproteinases (MMPs) and vascular endothelial growth factor (VEGF).(5) These inflammatory and proangiogenic factors are believed to derive from the ectopic endometrial tissue or from the response to that tissue.

Osteopontin(OPN) is a 70-kDa secreted phosphorylated glycoprotein that was originally isolated from bone matrix.(6) In human tissues, osteopontin has been found to be produced by epithelial cells of the gastrointestinal, urinary tracts, reproductive tracts, gall bladder, pancreas, lung bronchi, lactating breast, salivary glands, and sweat ducts.(7) Osteopontin is also secreted by activated macrophages, leukocytes, and activated T lymphocytes, and is present in extracellular fluids, at sites of inflammation, and in the extracellular matrix of mineralized tissues.(8) Osteopontin was found to be overexpressed in the tumors and serum of women with

ovarian cancer and was correlated with progression.(9, 10) Recent studies have shown that the overexpression of osteopontin was also related with breast cancer evolution and metastasis.(11) However, there are only limited data concerning the relationship between osteopontin and endometriosis. In this study, we measured mRNA expression of osteopontin in eutopic endometrium and plasma osteopontin levels in patients with endometriosis to evaluate the clinical value of this protein in the diagnosis of endometriosis.

II. **Materials and Methods**

1. Patients selection

One hundred-four women aged 17 to 50 years participated in this study with their written informed consent. The study was approved by the Institutional Review Board of Yongdong Severance Hospital and Cheil General Hospital. All patients underwent laparoscopy or laparotomy for different indications including pelvic masses, pelvic pain, suspicious endometriosis, infertility, and diagnostic evaluation. Post-menopausal women, previous hormone or GnRH agonist users and patients who had adenomyosis, endometrial cancer, endometrial hyperplasia or endometrial polyps, tubo-ovarian abscess and pelvic inflammatory disease were excluded. Endometriosis

was confirmed by pathological examination of biopsied tissue, and the extent of disease was staged according to the revised American Society of Reproductive Medicine (ASRM) classification of endometriosis.(12) Only after pathologic confirmation were patients assigned to the endometriosis group and the severity of the disease were classified as minimal-to-mild disease (Stage I and II) or moderate-to-severe disease (Stage III and IV). 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. The endometrial dating was further classified into five phases of the menstrual cycle (menstrual, early-, late-proliferative and early-, late-secretory phase).

2. RNA extraction and SYBR green real-time polymerase chain reaction (PCR)

Fresh surgical specimens were collected via endometrial biopsy. Biopsies were performed during exploration laparotomy or operative laparoscopy. Endometrial tissues were flushed with phosphate-buffered saline (PBS) and normal saline until contaminating blood was removed and stored at -70℃ until use.

Total RNA was extracted using the RNeasy Mini kit (Qiagen, Valencia, CA) and then total 2 ug RNA from each sample was reversibly transcribed into cDNA by SuperScript ™ III first-strand synthesis system (Invitrogen, Carlsbad, CA) according to manufacturer's protocols. The expression of candidate genes mRNA was measured

by SYBR green real-time PCR using ABI 7300 instrument (Applied Biosystems, Forster, CA). The specific primers for OPN (forward primer 5'-TCACCTGTGCCATACCAGTTAAA-3', reverse primer 5'-TGGGTATTTGTTGTA AAGCTGCTT-3') were designed. The PCR condition was performed in 20 $\mu\ell$ buffer containing 2 $\mu\ell$ cDNA, 5 pM each primer and power SYBR green PCR master mix (Applied Biosystems, Forster, CA). The thermal cycling conditions was pre-incubation for 2 min at 50℃ then denaturation for 10 min at 95℃ followed by 40 cycles of denaturation for 15 sec at 95℃ then annealing and extension for 1 min at 60℃. The normalization formula is target amount = $2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T$ = [C_T (Candidate gene) - C_T (Candidate gene GAPDH)] - [C_T (CONT-180) - C_T (CONT-180 GAPDH)].

3. Plasma Osteopontin Measurements

Blood samples were collected by peripheral venous puncture before anesthesia and immediately centrifuged at 1500*xg* at 4°C for 10 minutes. The separated plasma was aliquoted and stored at - 80°C for future analysis. Plasma concentrations of OPN were measured using specific commercial sandwich enzyme-linked immunosorbent assays (ELISA) according to manufacturer's protocols (Immuno-Biological Laboratories [IBL], Gunma, Japan). Results were expressed in nanogram per milliliter (ng/mL).

4. Statistical Analysis

Comparisons of osteopontin levels within and between the endometriosis and control groups were made using the Student's *t* test or Mann–Whitney *U* test where appropriate. The correlations between or within groups were evaluated with the use of Pearson's correlation coefficient or the Spearman rank correlation coefficient. Sensitivity and specificity for osteopontin were assessed by using receiver operating characteristic (ROC) curves.(13) 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 area under the curve of 0.5 indicates classifications assigned by chance. The statistical analysis was done by using the SPSS 12.0 package (SPSS Inc, Chicago, Il.) and statistical significance was accepted at $p<0.05$.

Ⅲ. Results

1. Clinical features

Of 104 patients, 64 patients had histologically confirmed endometriosis and 40 patients participated as controls. The mean age of the endometriosis patients and the controls were 33.11±7.71 years(mean ± SD) and 36.03±8.63 years (mean ± SD), respectively and the differences were statistically insignificant. (*P*=0.078, Table

1). The mean gravidarum was significantly higher in the control group than the endometriosis group (2.67±1.72 vs. 1.38±1.55, *P*<0.001), where serum CA-125 levels were significant higher in the endometriosis patients than the controls (76.14±105.58 IU/ml vs. 30.22±33.20 IU/ml, *P*=0.004).

Table 1. Clinical characteristics of study groups

	Endometriosis group (n=64)	Control group (n=40)	*P* value
Age (yr)	33.11±7.71	36.03±8.63	0.078
Gravidarum	1.38±1.55	2.67±1.72	<0.001
Parity	0.80±0.98	1.18±0.98	0.059
BMI(m^2/kg)	21.17±6.62	21.24±2.73	0.949
Serum CA-125	76.14±105.58	30.22±33.20	0.004

Note: Values are expressed as mean±SD
BMI=body mass index
Student t-test was used for statistical analysis

2. Expression of Osteopontin mRNA

RNA extraction and real-time polymerase chain reaction (PCR) were performed from 28 endometrial tissues from endometriosis patients and 29 endometrial tissues from the controls. In all endometrial tissue samples,

osteopontin mRNA was detected and based on the $\Delta\Delta C_T$ relative to the healthy endometrial tissue, CONT-180, the relative expression levels of OPN mRNA in the endometrial tissues of endometriosis patients were calculated. The mean OPN mRNA expression (mean±S.E.) was significantly higher in endometrial tissues of women with endometriosis than in endometrial tissues of controls (783.95±256.45 vs. 83.37±34.47, *P*=0.029, Figure 1). When OPN mRNA expression of endometrial tissue was analyzed according to different phases, late-proliferative phase showed the maximum expression in endometriosis group and menstrual phase showed the maximum expression in control group. (Table 2) The minimum expression was noted in the menstrual phase for the endometriosis group and early secretory phase for the control group. However, no significant differences were noted between OPN mRNA expression levels according to the menstrual phase in both groups. When OPN mRNA expression levels of the endometriosis patients and the controls were compared in each menstrual phase, only significant difference was noted at late proliferative phase, where the mean levels in endometriosis patients were significantly higher than those of controls. (1746.64±1148.12 vs. 16.37±8.66, *P*=0.024, Figure 2).

Figure 1. The expression of Osteopontin mRNA in endometrium of endometriosis patients and the controls

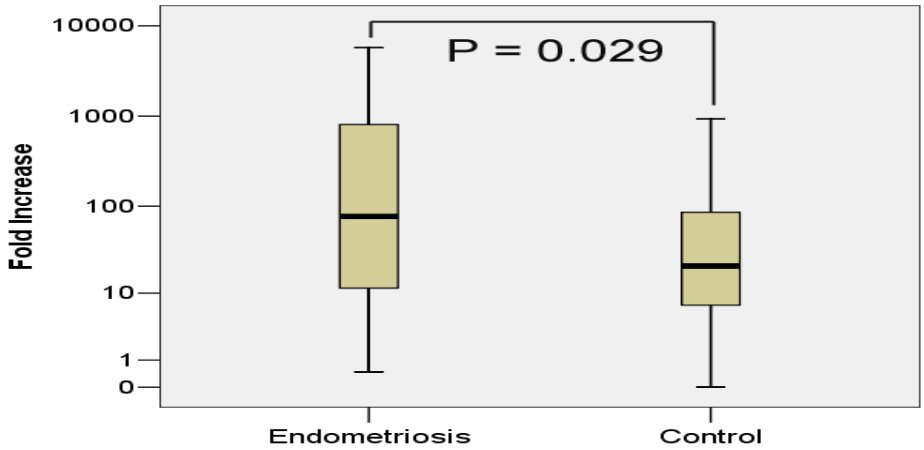


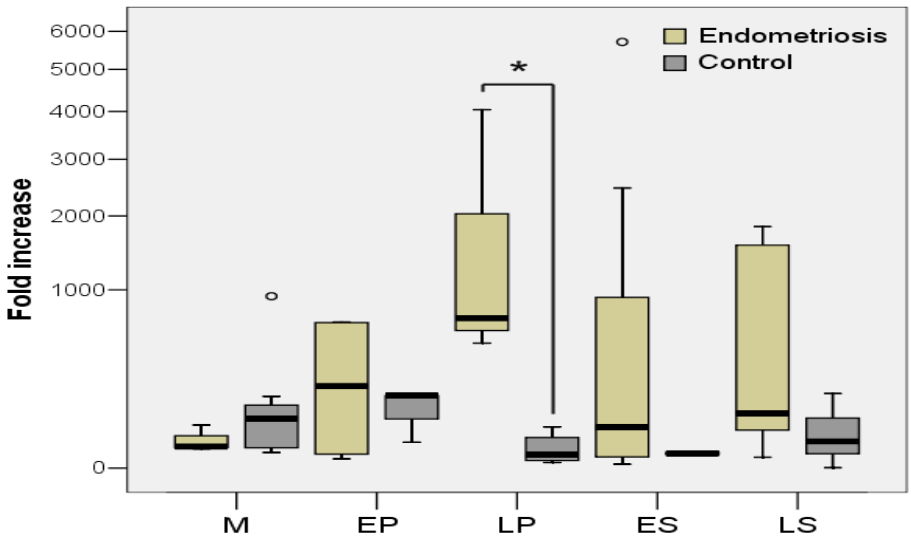
Table 2. The expression of Osteopontin mRNA in endometriosis patients group and the control group according to the menstrual cycle

	Endometriosis group (n=28)	Control group (n=29)	*P* value
All phases	783.95±256.45	83.37±34.47	0.029
Menstrual Phase	28.25±15.15	184.98±125.85	0.517
Early Proliferative Phase	336.54±190.38	117.28±48.14	1.000
Late Proliferative Phase	1746.64±1148.12	16.37±8.66	0.024
Early Secretory Phase	1056.68±641.63	6.89±0.85	0.582
Late Secretory Phase	641.09±278.47	54.69±20.78	0.113

Note: Values are expressed as mean±S.E.

Mann-Whitney test was used for statistical analysis

Figure 2. The expression of Osteopontin mRNA in endometriosis patients group and the control group according to the menstrual cycle



Note: M = menstrual phase, EP = early proliferative phase, LP = late proliferative phase, ES = early secretory phase, LS = late secretory phase

* $P < 0.05$ using Mann–Whitney *U* test

3. Plasma Osteopontin levels

Figure 3 shows plasma OPN levels in patients with endometriosis and the controls. The mean ± S.E. levels of plasma OPN in patients with endometriosis and the controls were 407.31±37.80 ng/ml and 160.07±18.94 ng/ml, respectively, and the differences were statistically significant ($P<0.001$). When plasma OPN levels were compared according to menstrual phase, plasma OPN levels of the endometriosis group were significantly higher during both proliferative and secretory

phase than the control group (Figure 4). However, in both groups, no significant differences of plasma OPN levels were noted between proliferative phase and secretory phase of the menstrual cycle (Figure 5).

Figure 3. Comparison of Plasma Osteopontin levels between endometriosis group and the controls.

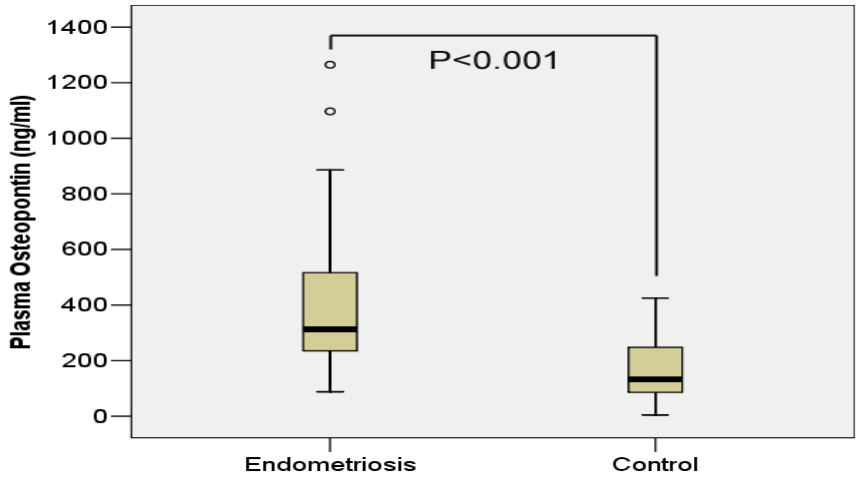
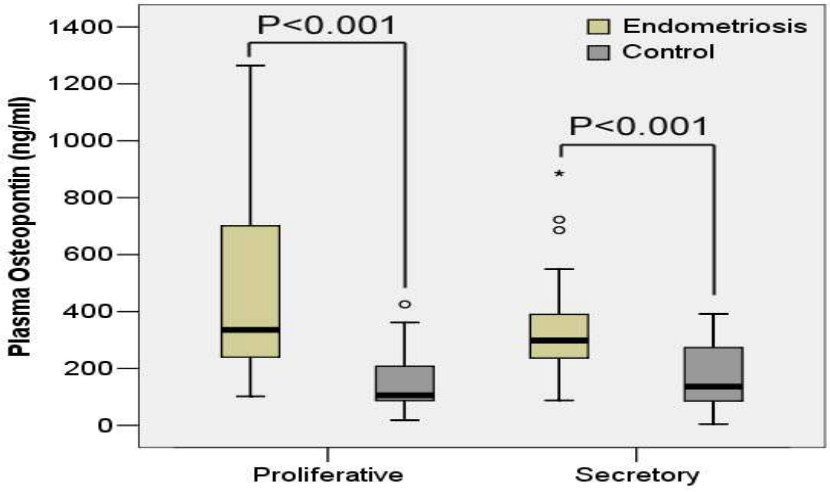
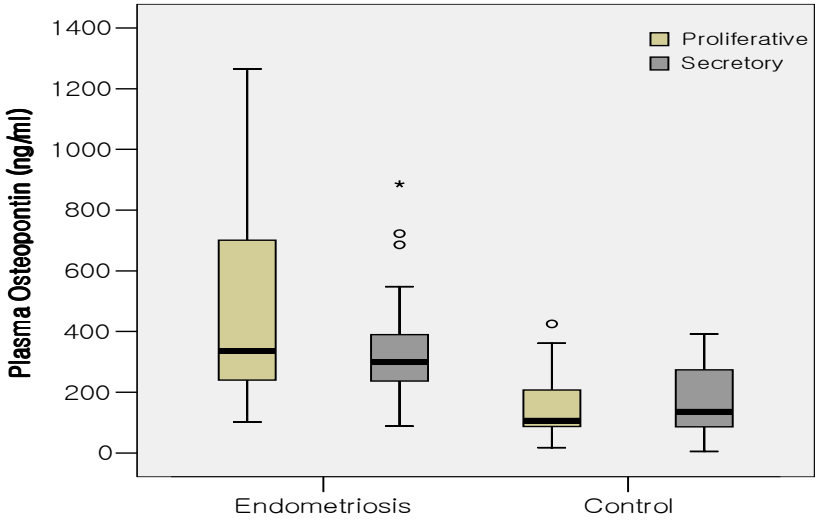


Figure 4. Comparison of Plasma Osteopontin levels in endometriosis group and the controls according to the menstrual cycle



Figre 5. Plasma Osteopontin levels in the study groups according to menstrual phase



4. Comparison of laboratory and clinical findings of the endometriosis patients according to the severity of the disease

Of 64 endometriosis patients, 16 patients were classified as minimal-to-mild disease (Stage I and II) and 48 patients were classified as moderate-to-severe disease (Stage III and IV). Comparison of the laboratory and clinical findings between minimal-to-mild disease and moderate-to-severe disease is shown in Table 3. No significant differences in age, gravidarum, parity, body mass index (BMI) and serum CA-125 levels were noted between the groups. OPN mRNA levels and plasma OPN levels were increased in moderate-to-severe disease, but no statistical significance was noted.

Table 3. Comparison of laboratory and clinical findings of the endometriosis patients according to the severity of the disease.

	Minimal-to-mild disease (N=16)	Moderate-to-severe disease (N=48)	*P* value
Age (yr)	32.00±6.98	33.48±7.97	0.511
Gravidarum	1.25±1.39	1.42±1.61	0.712
Parity	0.56±0.82	0.88±1.02	0.272
BMI(m^2/kg)	23.28±12.62	20.47±2.42	0.389
Serum CA-125	41.69±36.72	84.75±115.33	0.209
rAFS score	3.43±4.99	45.47±28.81	<0.001
Osteopontin mRNA levels*	777.82±441.54	786.86±323.01	0.987
Plasma Osteopontin (ng/ml)*	322.90±49.14	430.12±45.70	0.122

Note: Values are expressed as mean±S.D.

*Values are expressed as mean±S.E

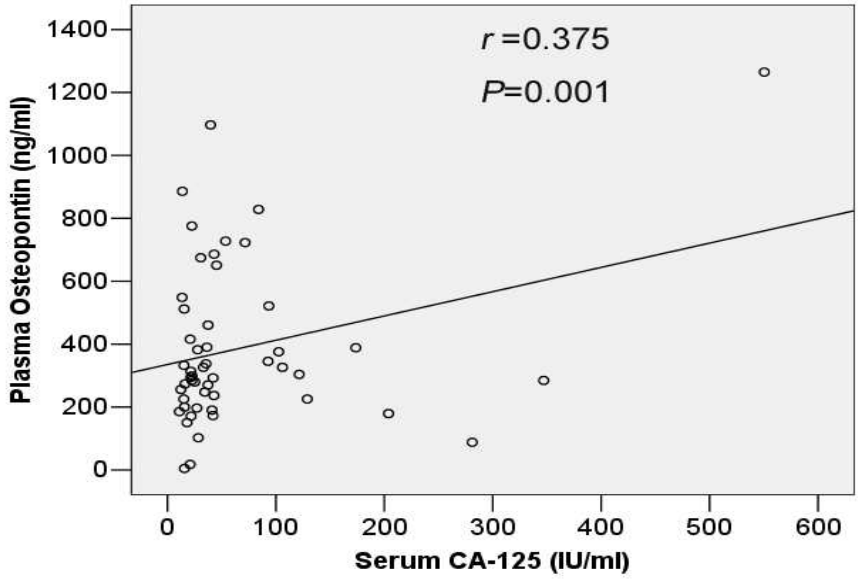
BMI: Body mass index, rAFS: revised American Fertility Society

5. Correlation of OPN mRNA levels, plasma OPN levels, serum CA-125 levels and stage of the disease.

When the correlation between OPN mRNA levels and plasma OPN levels were evaluated, no significant correlation was found (*P*=0.138). Plasma OPN levels did not show significant correlations between the severity of the disease nor revised

American Fertility Society scores (rAFS scores), but were found to have strong positive correlation between serum CA-125 levels (r=0.375, p=0.001, Figure 6).

Figure 6. Correlation between serum CA-125 levels and plasma Osteopontin levels

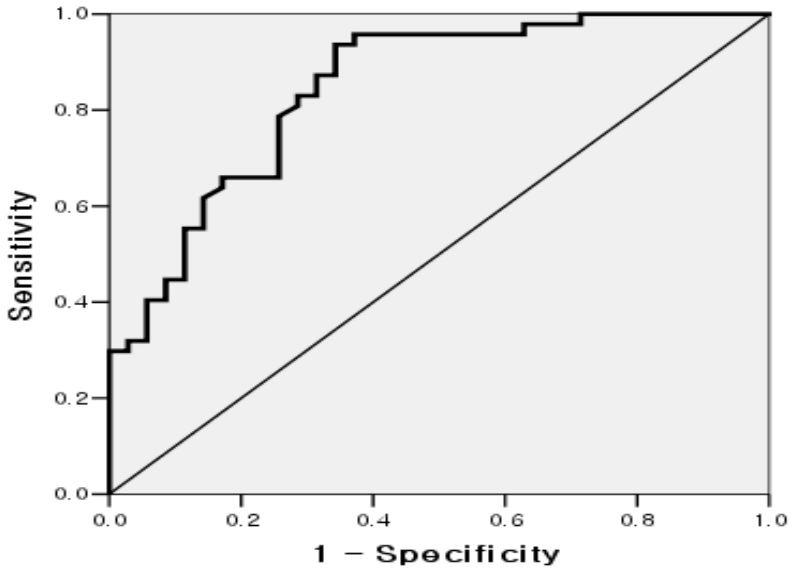


6. Sensitivity and specificity of plasma osteopontin in detecting endometriosis

To evaluate the utility of plasma OPN in diagnosing endometriosis, we examined the receiver operating characteristics (ROC) curve (Figure 7). For plasma OPN, the AUC was 0.846 (95% CI: 0.761-0.931, *P*<0.001). The optimal cut-off value was 167.68 ng/ml with sensitivity and specificity of 93.6% and 65.7%,

respectively. When the diagnostic performance of serum CA-125 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 0.834 (95% CI: 0.750-0.917, *P*<0.001) with sensitivity of 56.7% and specificity of 90.0%.

Figure 7. Receiver operating characteristic curves of plasma osteopontin for the differential diagnosis between endometriosis and controls.



Ⅳ. Discussion

In this study, we demonstrated that the expressions of OPN mRNA in eutopic endometrium of endometriosis patients are increased in comparison to those

without endometriosis and that circulating plasma OPN levels are higher in women affected by endometriosis. Plasma OPN levels showed positive correlation with serum CA-125 and displayed 93.6% sensitivity and 65.7% specificity with cutoff value of 167.68 ng/ml in detecting endometriosis.,

To our knowledge, there are only limited data on the relationship between OPN and endometriosis and no data has been published concerning mRNA expression of OPN in eutopic endometrium and plasma OPN levels in patients with endometriosis. Previous study with immunohistochemistry of osteopontin in endometriosis patients showed that OPN was stained more prominently in glandular epithelium than in interstitial space, and the staining pattern of OPN in ectopic endometriotic lesions was very similar to that in eutopic normal human endometrium in the secretory phase, suggesting important roles for OPN in the pathogenesis of endometriosis.(14)

Various evidences show that OPN is involved in the inflammatory process. OPN is known to be one of the most abundant proteins expressed by macrophages and is a potent macrophage-chemotactic stimulus. OPN appears to regulate macrophage infiltration during the inflammatory response. (15) OPN may alter chronic inflammatory responses as well, and data suggest that OPN may be particularly important in promoting retention of macrophages at sites of chronic inflammation.(16) Since, endometriosis is a pelvic inflammatory process with altered

function of immune-related cells in the peritoneal environment including increased macrophage concentration and activity, we assumed that OPN may be involved in the pathogenesis of endometriosis. Our findings showed that plasma OPN levels are increased in the patients with endometriosis. When the diagnostic performance of OPN was evaluated, the AUC was 0.846, which was higher than that of serum CA-125 (0.834) used in this study. The serum CA-125 in this study with a cut-off point of 35 IU/mL, which is the most generally reported in the literature, showed sensitivity and specificity of 56.7% and 90.0%, respectively.

In this study, the expressions of mRNA in eutopic endometrium of the endometriosis patients were also increased than the controls. Previous studies have characterized the expression of OPN in normal human endometrium and have shown that OPN is expressed at low concentration during the proliferative and early secretory phase of the menstrual cycle and increased in the mid to late secretory phase, suggesting a role in the regulation of endometrial function and implantation.(17,18) High density cDNA microarray screening confirmed more than 10-fold up-regulation in total endometrium between the early and mid secretory phases. (19) In this study, mRNA expressions of the eutopic endometrium were significantly higher then the controls. Although increased expressions were noted during late proliferative and early secretory phase of the menstrual cycle, no significant cyclic differences were noted. Not much is known about overexpression of OPN in the eutopic endometrium

of endometriosis. However, our findings support the idea that intrinsic abnormalities in the eutopic endometrium may play a role in the pathogenesis of endometriosis. Because OPN is a secreted protein that is related to cell adhesion and migration, we assumed that OPN may be involved in the attachment endometrium to peritoneal cavity and furthermore in invasion of endometrium.

Although previous studies indicated cyclic differences of OPN expression in normal endometrium , our findings supported the results of other studies indicating that there were no significant changes of plasma OPN levels according to the menstrual cycle.(8, 20) In this study, plasma OPN levels were significantly increased in comparison to the controls during both proliferative and secretory phase. Since the serum CA-125 levels are known to be affected by the timing of the blood collection in relation to the menstrual cycle, the fact that plasma OPN blood levels do not reflect hormonal changes over the menstrual cycle make this marker more clinically useful.(21)

V. Conclusion

In this study, we demonstrated that the expressions of OPN in eutopic endometrium and plasma OPN levels are increased in the patients with endometriosis. Fairly high sensitivity shown by the ROC curve suggests a possibility of a potential biomarker in the diagnosis of endometriosis. Further studies with large number of patients are

warranted for determination of exact role of OPN in the pathogenesis of endometriosis and clinical usefulness of plasma OPN, alone and in combination with other markers, in the diagnosis of endometriosis.

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국문 요약

자궁내막증 환자의 자궁내막 조직과 혈중 osteopontin의 발현

(지도교수 이 병 석)
연세대학교 대학원 의학과
안 영 선

목적: 본 연구는 자궁내막증 환자의 자궁 내막조직과 정상 대조군의 자궁내막 조직에서 세포간 부착에 관여하는 당 단백질의 하나인 osteopontin(OPN) mRNA의 발현을 알아보고, 혈중 Osteopontin level을 분석하여, 두 군간의 발현의 차이가 있는지 알아보고자 시행되었다

재료 및 방법: 개복 또는 복강경 수술을 통하여 조직학적으로 확진된 64명의 자궁내막증 환자와 자궁내막증이 없는 것으로 확인된 40명이 실험에 참가하였다. 개복 또는 복강경 수술 시 자궁내막 조직이 생검 되었고, 마취 후 정맥혈 채취를 통해 plasma를 검사하였다. 자궁내막조직의 OPN mRNA의 발현은 실시간 역전사 중합효소연쇄반응 (Real time quantitative RT-PCR)을 이용하여 비교하였고, plasma의 OPN의 농도는 specific commercial sandwich ELISA (enzyme-linked immunosorbent assays, Immuno-Biological Laboratories [IBL], Gunma, Japan) kit를 사용하였다.

결과: 자궁내막증 환자에서 자궁내막조직의 OPN mRNA의 발현은 정상 대조군보다 높게 나타났다 (783.95±256.45 vs. 83.37±34.47, P=0.029). 또한 자궁내막증 환자의 평균 plasma OPN은 407.31±37.80ng/ml으로 정상 대조군의 160.07±18.94ng/ml보다 통계학적으로 의의있게 높게 나타났다($P < 0.001$). Plasma OPN은 혈중CA-125와 상관관계를 보였으나 (r=0.375, p=0.001), plasma OPN 이나 자궁내막증의 중증도와는 상관 관계를 보이지 않았다. Plasma OPN과 자궁내막증의 예측정도는 ROC analysis상 AUC 0.846으로, cut off value를

167.68 ng/ml으로 하였을 때 sensitivity 93.6%, specificity 65.7%를 보여주었다.

결론: 본 연구 결과에 의하면 자궁내막증 환자에서 자궁내막의 OPN mRNA 표현과plasma의 OPN level은 증가되어 있었다. 따라서 OPN이 자궁내막증의 발생에 있어 조직에의 부착이나 침윤에 역할을 할 것으로 생각되고, 또한 증가된 plasma OPN은 자궁 내막증의 biomarker로서의 가능성도 보여준 것으로 생각된다.

핵심 되는 말: 자궁내막증, osteopontin, m-RNA, 자궁내막, plasma