Detection of endometriosis using immunocytochemistry of P450 Aromatase expressions in eutopic endometrial cells obtained from menstrual sloughing: A diagnostic study

Tita Husnitawati Madjid
Universitas Padjadjaran
Jumadi
Universitas Padjadjaran
Raden Tina Dewi Judistiani
Universitas Padjadjaran
Bethy Suryawathy Hernowo
Universitas Padjadjaran
Ahmad Faried (faried.fkup@gmail.com)
Universitas Padjadjaran https://orcid.org/0000-0003-2967-8125

Research note

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Abstract

Objective. To explore the possibility of a new diagnostic approach of endometriosis based on immunocytochemistry scoring of Aromatase P450 expressions in endometrial cells collected from menstrual sloughing. This is a case control study. Immuncytochemistry scores vs. histopathological examination in one tertiary- and secondary-level hospital in Bandung; two secondary level hospital in Garut and Sumedang, West Java, Indonesia. Thirty five patients with and without endometriosis were enrolled. All subjects had diagnostic procedures for endometriosis suspicion, with addition menstrual blood samples for cytopathological examination. The specimens were sent for immunocytochemistry assessment of P450 Aromatase expressions (ICAPEC). The previous procedure resulted in cut-off point of histo score (H-score), sensitivity, specificity, (+) and (-) ICAPEC predictive value.

Results. The P450 Aromatase expression in endometrial cells of women with endometriosis was significantly stronger than without one. The cut-off point of H-scores to detect endometriosis was >4. By this criteria, H-score had 94.6% sensitivity, 90.9% specificity, 92% positive predictive value and 93% negative predictive value. Immunocytochemistry scoring of Aromatase P450 expression in endometrial cells (ICAPEC) derived from menstrual blood specimen was a good candidate as alternatives approach in diagnostic procedure of endometriosis. Application and evaluation in clinical practice would provide the economically benefit in diagnostic procedure.

Introduction

Endometriosis is endometrium-like tissue outside uterus with similar response towards steroid hormone.[1–4] Several chronic symptoms such as dyspareunia, dys-menorrhea, poly-menorrhea, oligo-menorrhea, pelvic pain and infertility are related to the disease. Endometriosis prevalence is approximately 5–10% of general population and it reached up to 50% in infertile women. The point prevalence of endometriosis (n = 6146, mean age 40.4 ± 8.0 years) was 10.8 per 1000 (95% CI 10.5–11.0). Women aged 40–44 years had the highest prevalence rate of 18.6 per 1000 (95% CI 17.7–19.5). Infertility was documented in 37% of patients. A total of 6045 patients were included in the cohort of newly-diagnosed endometriosis (mean age 34.0 ± 8.1 years), corresponding to an average annual incidence rate of 7.2 per 10.000 (95% CI 6.5–8.0).[5] Another issue of endometriosis is the occurrence of several disturbances that can disturb the quality of life and fertility caused by accession, change of peritoneum function, hormonal, immunology and high relapse occurrence.[6]

It has been long agreed that the growth of endometriosis is hormone dependent. This is supported by the evidence of estrogen and progesterone receptor in endometriosis epistle and stromal.[7] Based on these, it is reasonable if the detection is addressed to cellular change assessment due to hormonal influences. Recently, aromatase enzyme occurrence in endometriosis implantation which affects estrogen biosynthesis which takes part in the development of endometriosis. In this case, estrogen is recognized as endometrial mitogen; therefore, the existence of estrogen production in ectopic endometrial itself is able to explain the failure of hormonal medication to some patients with endometriosis. Moreover, the

appearance of aromatase in endometriosis implantation supports the assumption that the production of local estrogen will develop the growth of endometriosis.[6]

Aromatase was discovered by Noble, Simpson, Johns and Bulun[8] in eutopic endometrium and endometriosis implantation in patients with endometriosis, whereas normal endometrium and peritoneum in women without endometriosis do not show the appearance of aromatase. It was also biochemically proven that eutopic endometrium and endometriosis implantation in patients with endometriosis is different from women without endometriosis.

Aromatase is found in eutopic endometrium tissue of endometriosis patients, adenomyosis and uterus myoma using immunohistochemistry technique and is not found in endometrium of cervical carcinoma patients without other gynecologic diseases. Aromatase found in endometriosis implantation and adenomyosis tissue. It is evident that this examination is very sensitive and specific. Our previous study shown endometrium cells that survive in menstrual blood can be isolated and analyzed for several proteins using immunocytochemistry technique. Immunohistochemistry and immunocytochemistry analyses are conducted based on histo score (H-score) through intensity and distribution of colored endometrium cells which is called semi quantitative examination.

The finding of aromatase enzyme in eutopic endometrium in women proven to suffer with endometriosis brought up the question whether aromatase activity can be found and assessed through the examination of menstrual blood of patients with endometriosis and whether there is significant difference in the appearance of aromatase between patients with and without endometriosis. If there is a significant difference and meaningful sensitivity, specificity values, positive and negative predictive values are found, then the appearance of aromatase in endometrium cells in menstrual blood can be used as an alternative diagnosis; we can conclude a theme of the main problem as follows: The growth of endometriosis depends on hormone especially estrogen as endometrium mitogen. Estrogen is not only produced in ovary but also produced locally in endometriosis implantation which plays a role in the growth and development of endometriosis and is assisted with the occurrence of aromatase activity.[9] The appearance of aromatase is also found not only in endometriosis implantation but also in eutopic endometrium of patients with endometriosis.[8, 10]

The available method of diagnosing endometriosis is by laparoscopic surgery. However, the procedure still faces problems since it is invasive and not all patients are willing to do it.[1] Other non-invasive methods have been brought up with detection of the appearance of P450 aromatase in endometrium cells that survive in menstrual blood of endometriosis patients. The aim of this study was to analyze the appearance of P450 aromatase based on the intensity and distribution in menstrual blood between patients with endometriosis and control. We hypothesized that there were significant differences in aromatase expression in menstrual blood in endometriosis patient compared to controls. If it expression has a diagnostic or predictive value, it can be used to detect endometriosis non-invasively in the early stages, so that the management of endometriosis can be done better. Furthermore, better management can also prevent further complications.

Materials And Methods

This is a case-control study that measure the P450 Aromatase in patient's menstrual blood with endometriosis by using laparoscopic or laparotomy surgery and supported with histopathology. The study was performed in Department of Gynecology and the Endocrinology Polyclinic of Fertility Reproduction Dr. Hasan Sadikin Hospital (RSHS), Bandung, Public Hospital in Sumedang and Ujung Berung, also from Dr. Slamet Hospital, Garut. The patients who had endometriosis were proven even further with histopathology examination.

Immunocytochemistry

The menstrual blood were centrifuged for 15 min. Immunocytochemistry smears were made on the object's glass. They were soaked in xylol and ethanol I, II and III, as well as in alcohol 90%, 80% and 90% for 5 min each. After that, they were washed with distilled water. The antigen unmasking retrieval procedure was done for 3x in 5 min, then cooled at room temperature for 15 min; washed with phosphate buffer saline (PBS). They were also incubated with 0.3% H_2O_2 in methanol for 10 min; washed with PBS. The smears were added the blocking reagents and were incubated for 5-10 min. They were given additional primary antibodies, incubated for 60 min; washed PBS 3x. Secondary antibodies were then added, incubated for 10 min; washed PBS 3x. Later on, streptavidin were used and were incubated for 10 min; washed PBS 3x. Chromogen was dropped and incubated for 10 min; washed with running water for 5 min. The smears were Incubated in mayerhematoxylin for 2 min; washed with running water after. The last steps of the procedure were to dehydrate alcohol 70, 80, 90% for 3 min and soak the smears into xylol for 3 min, then mounting.

Assessment of Immunocytochemistry

Histopathological endometriosis criteria: found endometrial gland epithelial cells and endometrial stromal in the tissue examined. The criteria for viable cells in this study were: there were cells or groups of stromal cells in their entirety in menstrual blood smear preparations. Generally, most cells will soon die when exposed to sunlight. Menstrual blood contains many proteolytic enzymes that are released from lysosomes which break due to a decrease in steroid hormones before menstruation, so that the menstrual blood cell component is easier to experience lysis. Lymphocyte cells around the group of stromal cells that are also stained with dark brown are used as positive controls.

Results

Patient characteristics and expression of P450 Aromatase in eutopic endometrial cells

Anamnesis, physical checkups, as well as other determinate check-related procedures were done alongside endometriosis and non-endometriosis patients based on the histopathology result. Next to be done was the analysis towards the performance of the P450 Aromatase in the menstrual blood. There were 5 samples that were not evaluable due to the lack of endometrium cells or no cell at all. The

samples that quantified as 37 cases of endometriosis and 33 cases of non-endometriosis. The endometriosis which have been found were on the stages of III and IV (severe). Based on the characteristics of the study subjects in Table 1, all variables of age, job, marital status and body mass index do not show significant differences between the endometriosis and control groups. Based on the homogeneity of data above, both groups were legible to be compared. Table 2 shown that endometrium cells in menstrual blood of patients with endometriosis displays the appearance of P450 Aromatase with strong, medium, weak and negative intensity by 25 (67.6%), 12 (32.4%), 0 and 0 cases while in control group there were 3 (9.1%), 9 (27%), 18 (54.5%) and 3 (9.1%) cases.

Endometrial cells in menstrual blood shown different Aromatase P450 expression intensities. The stronger the color produced shows the stronger intensity. Cell distribution was assessed based on the number of cells stained in each field of view are shown (Figure 1). Table 2 shown the distribution of the appearance of P450 Aromatase in patients menstrual blood with endometriosis by >80%, 50-80%, 20-50%, <20% and negative from each of 13 (35.1%), 17 (45.9%), 7 (18.9%), 0 and 0 cases while in control group, each group shows 2 (9.1 %), 2 (9.1%), 11 (33.3%), 15 (48.5%) and 3 (9.1%) cases, respectively. The difference in the appearance of P450 Aromatase (H-Score) between the two groups is significant. Receiver operating characteristics (ROC) Curve is used to decide the cut off point of the appearance of P450 as follows:

With cut off point >4, it is found that the sensitivity is 94.6% and the specificity is 90.9%. Using 2x2 templates, it is found that positive predictive value is 92% and negative predictive value is 93%. With a positive predictive value of 92%, it can be assumed that when the appearance of Aromatase found in endometrium cells in menstrual blood is positive, then the chance of a woman to suffer from endometriosis is 92%. With a negative predictive value of 93%, it can be assumed that when the appearance of Aromatase found in endometrium cells in menstrual blood is negative, then the chance for a woman not to suffer endometriosis is 93%.

Discussion

The analysis of the characteristics of the research subjects covers age, job, marital status, social and economy status, and body mass index, which shows that there is no significant difference ($p = 0.576$) in both groups; therefore, they are able to be compared. In this study, it is shown that the appearance of P450 Aromatase in menstrual blood of patients with endometriosis differs significantly compared to control. In patients with endometriosis, the appearance of P450 Aromatase shows cases with medium intensity of 32.4% and strong intensity of 67.6% (OR 6.25; 95% CI 1.21–36.25) and cases with negative and weak intensity were not found (Table 2).

The distribution of P450 Aromatase in patients with endometriosis also differs significantly compared to control with the distribution of 20–50% in 18.9% cases, 50–80% in 45.9% cases (OR 13.36; 95% CI 1.93-118.02) and > 80% in 35.1% cases (OR 10.21; 95% CI 1.43–92.35); case with negative distribution and < 20% was not found. In control shown different expression and 9.1% cases with negative result; 3.0%

cases were found negative result with the distribution > 80% (Table 2). Our result shown 91% sensitivity, 100% specificity, 100% positive- and 72% negative-prediction value. Another study also acquired a result of 100% sensitivity, 75% specificity, 86.9% positive- and 100% negative-prediction value. Our study shown 94.6% sensitivity, 90.6% sensitivity, 92% positive- and 93% negative-prediction value. The findings of the P450 Aromatase expression that obtained from menstrual blood by conducting immunocytochemistry examination and done non-invasively can be used to support the diagnosis for the establishment of endometriosis diagnosis.

Until recently, the main causes of endometriosis are still unknown. There are a lot of theories mentioned that plays a role in the pathogenesis of endometriosis. Retrograde menstruation occurs in 76–90% women. The prevalence of endometriosis which is far lower as 6.2–8.2% shows other factors in determining the susceptibility towards endometriosis. The response of aberrance immune that shows the inadequacy of menstrual reflux debris is one of the possible factors that tempted the occurrence of endometriosis other than genetic factor.

Conclusion

There were differences in the intensity and distribution of P450 Aromatase in menstrual blood of patients with and without endometriosis. ICAPEC derived from menstrual blood specimen was a good candidate as alternative approach in diagnostic procedure of endometriosis. Application and evaluation in clinical practice would provide the economical benefit of immunocytochemistry in diagnostic procedure

Limitation Of The Study

The limitation to this study is that the subjects do not represent all; only stage III and IV (severe).

Abbreviations

ICAPEC
Immunocytochemistry assessment of P450 Aromatase expressions
H-score
Histo score
RSHS
Dr. Hasan Sadikin Hospital
PBS
Phosphate buffer saline
ROC
Receiver operating characteristics

Declarations

Ethics Approval and Consent to Participate. This study protocol was approved by Faculty of Medicine Universitas Padjadjaran Ethics Committee Review Board, with number 138/FKUP-RSHS/KEPK/Kep/EC/2009; all study participants gave informed consent and agree to participate.

Consent to Publish. All authors declare that written informed consent was obtained from each patient details for publication of this study and accompanying images to be published.

Availability of data and materials section. Authors declare that the data will not be shared since they are patients' confidentiality.

Competing Interests. Authors have declared that no competing interests exist.

Authors' contributions. THM, J and RTDJ had examined, treated, observed and followed up the subject of this study. BSH had read and interpreted immunocytochemistry results. AF had corrected and written the manuscript.

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Tables

Table 1. Comparison of study subjects' characteristics

Characteristics	Group		*p* value
	Endometriosis (n=37)	Non endometriosis (n=33)	
Age (in years)			
mean (SD)	32.9 (6.9)	31.8 (9.0)	p = 0.576*
median	34.0	31.0	
min-max	20-49	17-54	
Occupation			
Housewife	23	21	p = 0.618**
Employed	14	12	
Marital Status			
Married	34	28	p = 0.355**
Single	3	5	
Social economy			
a. Upper	0	4	p = 0.060**
b. Middle	17	10	
c. Low	20	19	
5. **BMI** (kg/m^2)			
Mean (SD)	22.8 (3.5)	22.7 (3.8)	0.882*

Note: *t test, **x^2 tst

Tabel 2. Subjects distribution based on H-scoring of ICAPEC and P450 Aromatase Endometrium Cells in Menstrual Blood of Patients with Endometriosis and Control

H scoring	Group				OR (95%CI)	*p* value
	Endometriosis		Control			
	n	%	n	%		
Negative	0	0	3	9.1	-	0.001*
Weak	0	0	18	54.5	-	
Medium	12	32.4	9	27	1.0	
Strong	25	67.6	3	9.1	6.25 (1.2-36.2)	
P450 Aromatase (%)						0.001*
Negative	0	0	3	9.1	-	
< 20	7	0	15	48.5	-	
20-50	17	18.9	11	33.3	1.0	
50-80	13	45.9	2	6.1	13.36 (1.93-118.02)	
> 80	13	35.1	2	6.1	10.21 (1.43-92.35)	

Note: *chi square test, H-scoring: histophatology score, ICAPEC= immunocytopathological expression of P450 aromatase in endometrial cells

Figures

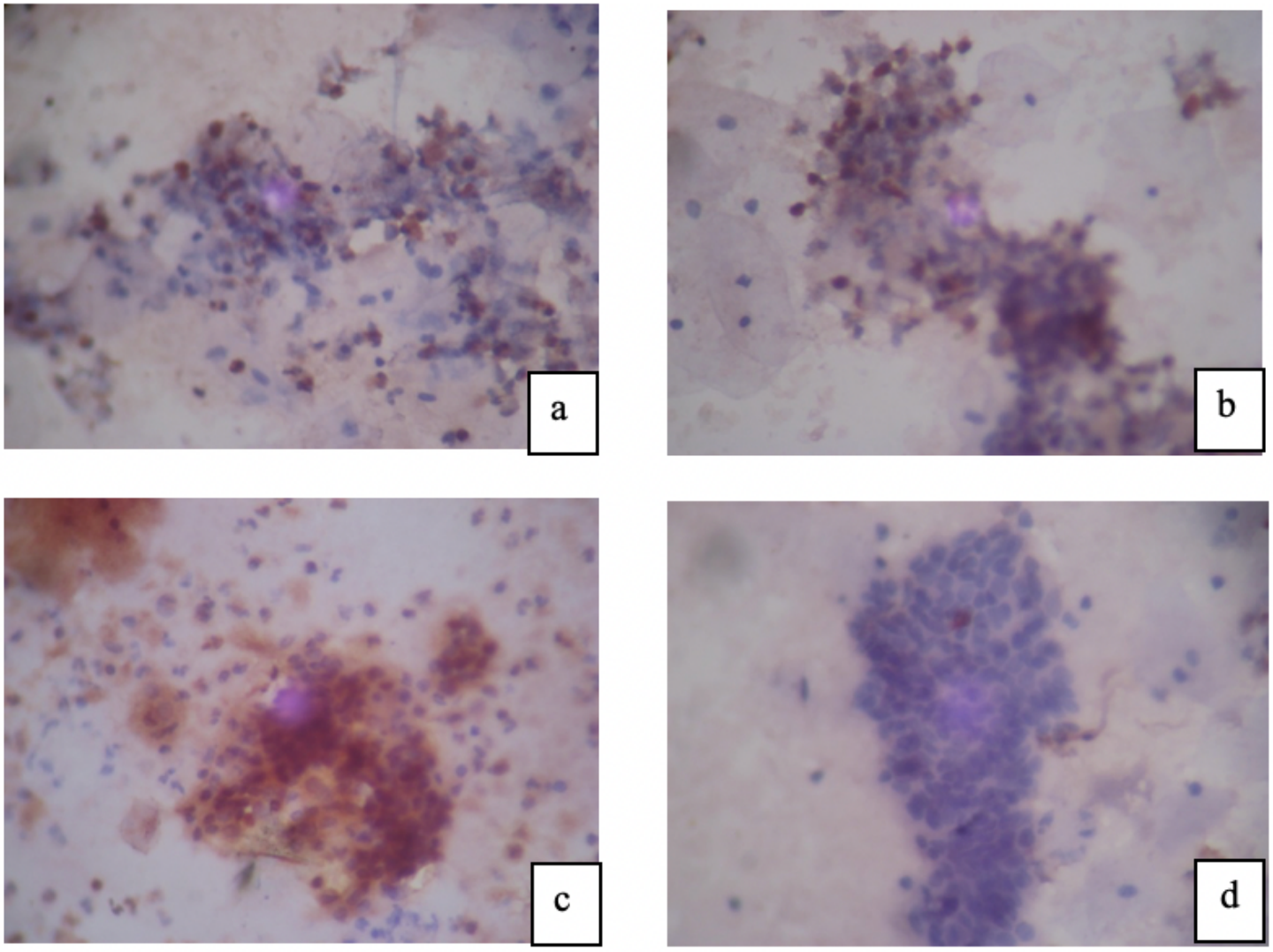

Figure 1

Figure 1. Immunocytochemistry of aromatase P450 in endometrial cell a. Aromatase P450 distribution of 20-50 % b. Aromatase P450 distribution of 50-80 % c. Aromatase P450 distribution of >80% d. Control (no expression of aromatase P450)