The 2nd Physics and Technologies in Medicine and Dentistry Symposium
IOP Publishing
IOP Conf. Series: Journal of Physics: Conf. Series 1073 (2018) 032081
doi:10.1088/1742-6596/1073/3/032081

Methylation analysis of plasminogen activator inhibitor-1 (PAI-1) gene in ovarian and peritoneal endometriosis

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Abstract. Endometriosis, defined as the growth of endometrial-like tissue outside the uterus, leads to the emergence of chronic inflammatory reactions, which are influenced by the plasminogen activator systems that are known to play a role in fibrinolysis. Hypofibrinolysis due to the excessive expression of the plasminogen activator inhibitor-1 (PAI-1) gene occurs in endometriotic cells. It has been known that the PAI-1 level is lower in normal endometrial cells than in endometriotic cells. The aim of this study is to assess the methylation level of PAI-1 in association with ovarian and peritoneal endometriosis tissues. This was a comparative cross-sectional study conducted on 13 women with ovarian endometriosis, 5 with peritoneal endometriosis, and 8 without endometriosis. DNA from the patient samples was isolated and subjected to bisulfite conversion. DNA methylation was observed by performing the methylation-specific polymerase chain reaction (MSP) method, followed by electrophoresis. The methylation level of PAI-1 was determined by measuring the band intensity using the ImageJ software. The results were statistically analyzed using the Kruskal–Wallis test. A two-tailed p value of <0.05 was considered to be statistically significant. Statistically significant difference was noted in the methylation levels of PAI-1 in ovarian endometriosis and peritoneal endometriosis samples compared with those noted in control samples (p = 0.006 and p = 0.003, respectively). The methylation levels in both sample types were lower than those in the control samples. However, the difference between the methylation levels of PAI-1 in peritoneal endometriosis and ovarian endometriosis were not statistically significant (p > 0.05). We found a low methylation level in the promoter region of PAI-1 gene, which led to an increase in the gene expression that may contribute as a risk factor in ovarian endometriosis and peritoneum endometriosis.

1. Introduction
Endometriosis is a chronic inflammatory disease that is characterized by the growth of endometrial-like tissue outside of the uterine cavity [1,2]. Endometrial-like tissue is typically present in the pelvic region, especially in the ovaries, but has also been detected in multiple organs such as the lungs, central nervous system, diaphragm, and peripheral and axial skeleton [3]. Endometriosis in the pelvic...
area can induce symptoms such as dyspareunia, dysmenorrhea, and chronic pelvic pain [2]. In addition, the prevalence of endometriosis is approximately 5% with the highest prevalence noted between 25 and 35 years of age [4]. Endometriosis may affect 35%–50% of women with infertility, pelvic pain, or both [5]. The incidence of endometriosis in Indonesia has not been determined owing to the lack of epidemiological studies on this disease.

Endometriosis is a complex multifactorial gynecological disease that affects women of childbearing age across the world [1-6]. Some basic biological functions, such as angiogenesis, immune response, fibrinolysis, and apoptosis, are impaired in abnormal endometrial tissues, which eventually develop into endometriosis [7]. The potential etiological factors of endometriosis-associated infertility are associated with inflammatory cytokines, growth and angiogenic factors, fibrinolysis, tissue remodeling, and tumor invasion [2-8]. The overproduction of prostaglandins, cytokines, and chemokines is associated with the presence of ectopic tissues in the peritoneal cavity, and this is another typical feature of endometriosis [9]. Endometriosis may be initiated by fibrin matrix persistence that allows the endometrial fragment to be deposited in peritoneal pockets [10].

PAI-1 is a member of the serine protease inhibitor family and a determinant factor in inhibiting the fibrinolytic activity of tissue-type plasminogen activator (tPA). This inhibition process involves the cleavage of fibrin produced by plasmin from plasminogen [8]. The modified fibrinolytic activity of women with endometriosis can result in the production of endometrial fragments, which possess high adhesion potential to the peritoneal lining [11]. Physiologically, PAI-1 and urokinase-type plasminogen activator (uPA) play the central role in angiogenesis, fibrinolysis, and wound healing [12]. Nevertheless, a non-physiologic elevation of PAI-1 may contribute to endothelial dysfunction and abnormal ovarian function and phenotype [13].

Changes in the PAI-1 transcription pattern could influence the changes in PAI-1 biosynthesis. In vitro studies have proven that guanine (G) deletion polymorphism in the promoter region of PAI-1 suggests a higher activity of the 4G allele than that of the 5G allele because the 5G allele contains an additional binding site for DNA-binding protein, which in turn acts as a transcriptional repressor [14,15]. The deletion of the allele activates gene transcription to elevate the plasma PAI-1 activity, which significantly diminishes the fibrinolytic activity (hypofibrinolysis) [16]. This finding agrees with that of a study conducted by Bedaiwy in 2006, who recorded an association between PAI-1 promoter polymorphism and endometriosis. PAI-1 promoter polymorphism has been associated with hypofibrinolysis, which is over-expressed in women with endometriosis [16,17]. PAI-1 level is higher in endometriotic cells than in normal endometrial cells [18]. There is an interesting difference in this expression to be studied because the change in the gene expression is regulated by epigenetic mechanisms such as DNA methylation.

There is still no information on the level of DNA methylation in PAI-1 in the peritoneal and ovarian endometriosis tissues that may affect the gene expression. Therefore, this study aimed to analyze the methylation level of PAI-1 in association with the ovarian and peritoneal tissues using methylation-specific PCR among Indonesian women.

2. Methods
Endometrial tissue samples were collected from 11 women aged 25–35 years with laparoscopically diagnosed and histologically confirmed ovarian endometriosis with their informed consent. Conversely, endometrial control tissue samples were collected from 8 healthy women who had undergone microcurettage and laparoscopy and were free of endometriosis. In addition, the peritoneal endometrial tissue samples were collected from 5 women with laparoscopically diagnosed and histologically confirmed peritoneal endometriosis. All tissue samples were subjected to bisulfite conversion and MSP methods. DNA from the tissue samples was extracted using the gSYNC™ DNA Extraction Kit and quantified by the Maestrogen Maestro Nano Spectrophotometer (USA).

DNA isolated from the samples was subjected to bisulfite conversion and MSP process. The methylation-primer sets for MSP were (Forward) 5’- AGT TAG GTA TGG TGGTAG GCG T -3’ and (Reversed) 5’ - CAA AAA AAT CAA AAA ATT AAA CGA – 3’. The unmethylation-primer were
(Forward) 5’ - TAG TTA GGT ATG GTA GGT GT -3’ and (Reversed) 5’ - AAA AAA ATC AAA AAA TTA AAC AAT – 3’. The MSP products were visualized by electrophoresis with 2.4% agarose gel containing ethidium bromide. The methylation levels in the samples were determined by measuring the band intensity using the ImageJ software. The degree of methylation in each sample was estimated from the area of the respective methylated band in comparison with the total area of the methylated and unmethylated bands.

The data obtained from these experiments were processed and statistically analyzed using the SPSS Statistics version 21.0 using the Kruskal–Wallis test to determine the statistically significant difference among the 3 sample groups. Next, a post-hoc analysis was performed using the Mann–Whitney U-test to compare the difference between the 2 independent groups. The two-tailed p value of <0.05 was considered to be statistically significant.

3. Results
The gel electrophoresis result of the MSP products of PAI-1 is shown in Figure. 1.

![Figure 1](image)

**Figure 1.** The gel electrophoresis results from the MSP product of PAI-1 gene. M = DNA marker, m = methylated, and u = unmethylated. K9 is control sample, O4 is ovarian endometriosis sample, and P2 is peritoneal endometriosis sample.

The mean methylation level of PAI-1 in peritoneal endometriosis was 23% ± 10%, whereas the median (max–min) methylation level of PAI-1 in ovarian endometriosis was 19% (96%–2.0%); in the normal endometrium tissues, the median (max–min) level was 97% (99–56%). A lower methylation rate was noted for ovarian endometriosis and for peritoneal endometriosis in comparison to that for normal endometrium tissues. Post-hoc analysis using the Mann–Whitney U-test revealed that the methylation percentage between the control and peritoneal endometriosis samples showed a statistically significant difference ($P = 0.003$). Moreover, the methylation percentage between the control and ovarian endometriosis samples was also statistically different ($P = 0.006$; Figure. 2).
The methylation percentage of PAI-1 in peritoneal and ovarian endometriosis as well as in normal endometrium tissues.

* = significance p < 0.05 (Mann–Whitney U-Test).

4. Discussion

Endometriosis is generally regarded as a complex trait, resulting from genetic and environmental causes [19]. The present data strongly suggest that epigenetic factors play a definite role in the pathophysiology and pathogenesis of endometriosis. In addition, DNA methylation can potentially be used as a diagnostic tool for predicting the recurrence risk of endometriosis [1]. Methylation, involving the addition of methyl groups to cytosine residues in the CpG region, is an important epigenetic process [20]. Methylation occurs in most CpG sites outside of the CpG island, whereas unmethylation occurs on most of the CpG islands in a gene promoter. In general, the administration of certain cytosine to the CpG island located in the methylated gene promoter region results in the gene being silenced by methylation. This process is also called as hypermethylation. Additionally, when a certain cytosine is not administered, the gene is not silenced by methylation; the process of methylation in the CpG island is called hypomethylation. When a promoter region which normally recognized by an activating transcription factor is methylated, its transcription gets inhibited. Several studies have successfully demonstrated the involvement of DNA methylation in a number of genes during the development of endometriosis, including HOXA10, ER-α, SF-1, and PGR [21].

PAI-1 belongs to the serine protease inhibitor family and becomes a determinant factor in inhibiting the fibrinolytic activity of the tPA [8]. Non-physiological elevation of the PAI-1 activity may contribute to abnormal ovarian function and endothelial dysfunction [13]. Polymorphism of the 4G allele has been associated with high levels of plasma PAI-1 antigen and PAI-1 activity. Another in vivo study demonstrated an association of the persistence of fibrin with the polymorphism of the 4G allele in the promoter region of PAI-1. Hypofibrinolysis, which can cause persistence of fibrin matrix in peritoneal pockets, allows the deposited endometrial fragments to initiate endometriosis. Thus, we can conclude that fibrinolysis is modulated by several factors and polymorphisms in PAI-1 are one of the important determinants. The polymorphism of the 4G allele in the promoter region of PAI
activates its gene transcription level to elevate the plasma PAI-1 activity toward diminishing the fibrinolytic activity (hypofibrinolysis). Conversely, individuals with the 5G/5G genotype, namely, those with efficient fibrinolysis, fail to build sufficient fibrin matrix to encourage the growth and invasion of trapped endometrial fragments deposited by retrograde menstruation. This genotype is related to the rapid clearance of retrograde menstrual materials and the failure of initiation of endometriosis [16]. This condition occurs in healthy women who do not suffer from endometriosis. The retrograde menstruation theory considered explaining the pathogenesis of the basic mechanisms of endometriosis development. This theory is associated with the coexistence of endometrial fragments and fibrin mesh. Fibrin formation is regulated by the proteolytic cleavage of fibrinogen to fibrin. Consequently, higher baseline levels of PAI-1, found in 4G allele, may deliver increasing amounts of PAI-1 in response to retrograde menstruation.

As mentioned, methylation is one of the most important epigenetic mechanisms involving the process of adding methyl groups to the 5th carbon position in the cytosine dinucleotide residue of DNA on the CpG island. The DNA methylation analysis in this study is the methylation-specific PCR (MSP) technique. MSP is an application of the bisulfite sequencing method. For a sequence in gene-containing CpG, the allele in which the CpG is methylated and the one in which it is not methylated must provide a different sequence after bisulfite modification. A specific primer comprising methylated (M) and unmethylated (U) primers was used for the PAI-1 promoter region. The M primer completes the methylated CpG sequence but not the unmethylated CpG sequence. Conversely, the U primer completes the unmethylated CpG sequence, but not the methylated CpG sequence. PCR only amplifies the sequence (allele) with the methylated CpG. Typically, the M and U primer pairs are both used for the same gene, PAI-1, and the amplified products are run side by side on the agarose gel for comparison. The result of MSP of PAI-1 with electrophoresis shows that the methylated and non-methylated promoter areas have been well amplified, as indicated by the appearance of a band at 197 bp for both M and U primers.

Our study demonstrates that the promoter area of PAI-1 in women with peritoneal endometriosis undergoes hypomethylation compared with that in women without endometriosis. Hypomethylation could cause hyper-expression of the gene. Yao [23] noted higher levels of PAI-1 in the ovarian endometriotic tissues than in normal endometrium [23]. In a study conducted by Sui et al. the levels of PAI-1 observed in endometriotic cells were higher than those in normal endometrial cells [18]. These data together suggest that the use epigenetic changes as biomarkers for endometriosis. Hypomethylation of specific chromosomal domains has also been linked to chromosome instability and altering gene expression [24]. In normal cells, the active PAI-1 expression is an unmethylated gene on the CpG island. The condition of the local PAI-1 hypomethylation on CpG island causes transcription of this gene. The transcribed PAI-1 leads to the formation of PAI-1 in the peritoneal and ovarian endometriosis tissues at levels higher than those in normal tissues. This finding is in accordance with that of a study conducted by Sui et al. [18] who clarified that endometriotic cells secreted more PAI-1 than endometrial cells.

This finding on hypomethylation in peritoneal endometriosis and ovarian endometriosis may be due to the retrograde menstruation theory, which is associated with fibrin formation. This finding is based on the theory that no abnormal fibrin formation occurs in the endometrial tissue because of the absence of hypofibrinolysis as a result of increasing PAI-1 activity in the controls (healthy women). In the endometriosis tissue, fibrin formation may occur owing to hypofibrinolysis as a result of an increase in the PAI activity due to gene polymorphism. In addition to its main function, PAI-1 may also play a role in endometriosis by facilitating the detachment of endometrial cells.

In contrast to the relationship of each case group (ovarian and peritoneal endometriosis) with controls, there was no significant difference in the relationship between ovarian and peritoneal methylation percentages of PAI-1 ($P > 0.05$). Both the groups demonstrated the occurrence of DNA hypomethylation of PAI-1. This could be because PAI-1 plays an important role in the endometriotic cell invasion. A low methylation percentage of PAI-1 in ovarian and peritoneal endometriosis patients may also corroborate the potential link between adhesion formation and the pathogenesis of
endometriosis. Our data demonstrate the association between low DNA methylation percent (hypomethylation) of PAI-1 and endometriosis (ovarian and peritoneal). Furthermore, this novel finding could form the basis of diagnostic test for women at risk for developing endometriosis.

5. Conclusion
The methylation levels of PAI-1 in ovarian and peritoneal endometriosis samples were low compared with those in control samples, and the methylation status between peritoneal and ovarian endometriosis were not significantly different.

Acknowledgments
This study was funded by Research Grant for Indexed International Publication for Student Final Assignment 2017 from The Research Council and Community Services of Universitas Indonesia.

Abbreviations
CpG : Cytosine-phosphate-guanine
MSP : Methylation-specific PCR
PAI : Plasminogen activator inhibitor-1
PCR : Polymerase chain reaction
tPA : Tissue-type plasminogen activator

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