Analysis of the methylation profiles of the steroidogenic factor-1 (SF-1) gene in peritoneal and ovarian endometriosis

N G Annisa¹, R R Febri², Darmawi¹, T Kinasih¹, R Muharam³,4 and Asmarinah²,5*

¹Undergraduate Program, Faculty of Medicine, Universitas Indonesia, Jakarta, 10430, Indonesia
²Human Reproductive, Infertility and Family Planning, IMERI, Faculty of Medicine, Universitas Indonesia, Jakarta, 10430, Indonesia
³Master Program in Biomedical Sciences, Universitas Indonesia, Jakarta 10430, Indonesia
⁴Obstetric and Gynecological Department, Faculty of Medicine, Universitas Indonesia/Cipto Mangunkusumo Hospital, Jakarta, 10430, Indonesia
⁵Medical Biology Department, Faculty of Medicine, Universitas Indonesia, Jakarta, 10430, Indonesia

*E-mail: asmarinah.si@ui.ac.id

Abstract. Endometriosis is a disease characterized by implantation of endometrial-like tissues outside the uterus. Until now, its pathogenesis remains unclear. Previous studies have suggested a possible role of epigenetics in endometriosis. The purpose of this study was to analyze the methylation profile of the SF-1 gene in peritoneal and ovarian endometriosis. This gene has a role in the synthesis of steroid hormones, which are thought to participate in development of the disease. This study used nine samples of ovarian endometrial tissues, five samples of peritoneal endometrial tissues and nine controls. Endometrial tissues were obtained from patients undergoing endometriosis surgery and controls were from patients undergoing microcurretage before in vitro fertilization. DNA from the samples were isolated, treated with sodium bisulfite, and then analyzed by methylation-specific polymerase chain reaction. Statistical analysis used to obtain conclusion was a Kruskal–Wallis test and followed by post hoc analysis with a Mann-Whitney U test. There is a significant difference between ovarian endometriosis, peritoneal endometriosis and controls with \(p \leq 0.001\). We further discovered a significant difference between control and peritoneal endometriosis (\(p=0.001\)) and between ovarian and peritoneal endometriosis (\(p=0.008\)). There was no significant difference between control and ovarian endometriosis (\(p=0.730\)). Our results suggest that changes in methylation profile of the SF-1 gene may be associated with the development of peritoneal endometriosis. The difference in methylation profiles of ovarian and peritoneal endometriosis might reflect different pathogenic mechanisms for both type of diseases.

1. Introduction
Endometriosis is a disease characterized by implantation of endometrial-like tissues outside the uterus. It is found in 10 to 15% of females of reproductive age with the peak of prevalence at ages 25 to 35 [1,2]. Endometriosis is diagnosed in 30 to 40% women with infertility and pelvic pain [1]. The most common location of implantation is in the pelvic peritoneum, but endometriotic tissues can also be found in ovaries and the rectovaginal septum and, more rarely, in the pericardium, pleura or brain [3].
The pathogenesis of endometriosis remains unclear. It has been described as a hormonal, immune and genetic disease, as well as a disease that is caused by exposure to environmental factors [4]. In 1920, Sampson proposed the retrograde menstruation theory, which explained the implantation of endometrial tissue outside the uterus as the result of backflow of menstrual blood. However, this theory could not explain the appearance of endometriosis in other rarer locations, such as the pleura, pericardium or brain. Another theory, colonic metaplasia, explained endometriosis in terms of de-differentiation of peritoneal tissues into endometrial cells, while metastatic theory explains hematogenous and lymph spreading as the cause of endometriosis in distant areas [5].

With regard to its description as a hormonal disease, endometriosis has been associated with the upregulation of estrogen in endometriotic tissues. The action of estrogen is mediated by various factors and genes and endometriotic lesions exhibit high estradiol biosynthesis and low inactivation of estrogen compared to normal endometrial cells. Estrogen itself is associated with the proliferation of endometrial cells. High levels of aromatase, a key enzyme in estrogen biosynthesis, have also found in endometriotic cells [6].

As a genetic disease endometriosis could be explained by the theory of epigenetic inheritance. Epigenetics is defined as stable inheritance without changes in DNA contents or sequences and involves DNA methylation, histone modification and transcription factor network. DNA methylation is one of the best understood mechanism in epigenetics and involves addition of methyl groups to specific dinucleotides sites within the genome. Hypomethylation of a promoter is associated with overexpression of a gene, while hypermethylation is associated with low expression of a gene [7].

Steroidogenic Factor-1 (SF-1) is a transcriptional factor involved in steroid hormone biosynthesis and is essential for the production of aromatase and steroidogenic acute regulatory protein (StAR), which functions to facilitate the entry of cholesterol into mitochondria [6]. SF-1 is usually undetectable in normal endometrial cell but is overexpressed in endometriotic tissues. This phenomenon is thought to be caused by hypomethylation of the SF-1 promoter [8].

Even though there has been previous research that studies the methylation profile of SF-1 gene in endometriotic cells, there is only small number of studies which differentiates the methylation profile of SF-1 gene between different type of endometriosis.

2. Material and Methods
Enzyme-treated tissues were obtained after informed consent from 14 women undergoing laparoscopic surgery and who had been histologically confirmed as having ovarian or peritoneal endometriosis. Nine were diagnosed with ovarian and five with peritoneal endometriosis. Controls were endometrial tissues obtained after informed consent from nine women undergoing microcurettage before in vitro fertilization and who had been histologically confirmed to be endometriosis-free.

The tissues were homogenized using a rotor and DNA was extracted using a Qiagen DNA Extraction Kit. Extracted DNA was treated with bisulfite conversion and then processed by methylation-specific PCR. PCR products were visualized by electrophoresis in 2.4% agarose gels containing ethidium bromide. Intensity of PCR product bands was analyzed using ImageJ software.

Using Shapiro-Wilk test, the data was found to have non-normal distribution ($P < 0.001$). Hence, Kruskal–Wallis test was used for statistical analysis, and was followed by a post hoc analysis using a Mann-Whitney U test. The tests compared percentages of methylation determined using the equation:

$$\text{percentage of methylation} = \frac{\text{intensity of methylated DNA band}}{\text{total intensity of methylated and unmethylated band}} \times 100\%$$

A $P$ value $< 0.05$ was considered to be significant.
3. Results
The methylation profile of SF-1 gene is shown as percentage methylation. Table 1 shows percentage of methylation in difference endometriotic groups obtained from this study. Both control and ovarian endometriosis groups have median of 100%, while the peritoneal endometriosis group has median of 87.5%. Electrophoresis results from the different groups are shown in Figure 1.

Table 1. Percentage of methylation in different endometriotic groups.

| Groups                      | N  | Median(Range) |
|-----------------------------|----|---------------|
| Percentage of Methylation   |    |               |
| Control                     | 9  | 100 (100–100) |
| Ovarian Endometriosis       | 9  | 100 (58.2–100) |
| Peritoneal Endometriosis    | 5  | 87.5 (0–90.3)  |

![Figure 1](image)

**Figure 1.** The gel electrophoresis result from the MSP product of SF-1 gene in control sample as well as in ovarian and peritoneal endometriosis samples. Ma = DNA marker; M = methylated band; U = unmethylated band.

The percentages of methylation between three groups were analyzed using a Kruskal–Wallis Test. *P* values obtained is <0.001 in all cases, which indicate there is a statistically significant difference in the distribution of methylation percentage between the three groups.

Post hoc analysis using a Mann-Whitney U test showed that there was a statistically significant difference in the SF-1 methylation profiles between the control and peritoneal endometriosis groups (*P* = 0.001) and between the ovarian and peritoneal endometriosis groups (*P* = 0.008). However, there was no significant difference between the methylation profiles of SF-1 gene between the control and ovarian endometriosis groups (*P* = 0.738).
Using data’s medians for comparison of the percentage of methylation, we found that there is a statistically significant hypomethylation of the SF-1 gene in peritoneal endometriosis.

4. Discussion
In this study, we analyzed the methylation profile of the SF-1 gene promoter in normal endometrial cells, ovarian endometriosis and peritoneal endometriosis.

Xue et al. identified a CpG island in promoter of the SF-1 gene and their study found an increase in methylation in the promoter region of normal endometrial cells [9,10]. This result is consistent with our study, where all endometrial tissues from the control group exhibited fully methylated DNA in the promoter region of the SF-1 gene.

Another study found hypomethylation in the SF-1 gene promoter in endometriotic cells. SF-2, a transcription factor found in abundance in endometriotic cells, binds to the unmethylated promoter of SF-1 and stimulates its expression [11]. This finding is consistent with our results, which found a statistically significant difference in the methylation profiles between control and peritoneal endometriosis. However, this finding is inconsistent with our observations in ovarian endometriosis where we found no significant difference between SF-1 promoter methylation profile in ovarian endometriosis and controls.

Most past studies have not differentiated by the location of endometriotic cells. The differential methylation profiles of the SF-1 gene described here might suggest different pathogenic mechanisms between ovarian and peritoneal endometriosis. The most widely accepted pathogenic theory is retrograde menstruation, where menstrual blood and tissues flow back through the fallopian tubes and attach to the peritoneum. There are three conditions that need to be met in order to consider retrograde menstruation as the cause of endometriosis. First, endometrial cells from menstrual flow must flow back through the fallopian tubes. Second, the endometrial cells must be viable and able to adhere and proliferate. Third, the anatomical position of the endometriotic lesion in the pelvic cavity must be correlated with the transplantation theory of exfoliated cells [12]. This theory is supported by laparoscopy findings that 76-90% of women with patent fallopian tubes demonstrated retrograde menstruation, and women with endometriosis have larger volumes of retrograde flows. Women with endometriosis demonstrate abnormalities in uterine contraction during menstruation and tend to have shorter menstrual cycle lengths and heavier periods, both of which could increase the retrograde menstrual volume. Moreover, women with Mullerian anomalies or obstruction in the vaginal or cervical outlet are at increased risk of developing endometriosis at an earlier age [13].

Retrograde menstruation and implantation theory is a suitable explanation of the pathogenesis of peritoneal endometriosis [12]. In retrograde menstruation and implantation of endometriotic tissues in the peritoneum, it was found that expression and enzyme activity of P450 aromatase was amplified by up to 1475 times. P450 aromatase is a key enzyme in estrogen biosynthesis, and its expression is induced by SF-1, a transcriptional enhancer that binds to a specific response element in the promoters of several steroidogenic genes. In normal endometrial cells, the fully methylated SF-1 promoter recruits a transcriptional suppressor, methyl-CpG-binding domain protein 2 (MeCP2) which prevents activation of the promoter. Meanwhile, in endometriotic tissues, the transcription factor upstream stimulatory factor-2 (USF-2) binds to an E-box element in the hypomethylated SF-1 promoter and activates it [14]. These findings could explain the result of hypomethylation in the SF-1 gene promoter found in peritoneal endometriosis.

Meanwhile, the cause of pathogenesis of ovarian endometriosis remains controversial. Aside from the adhesion of menstrual debris from retrograde menstruation, there is another theory for pathogenesis of ovarian endometriosis: coelomic metaplasia. This theory postulates that there is metaplastic potential in pelvic mesothelium that might convert normal cells to endometriotic cells. This theory has also been accepted for pathogenesis of several ovarian tumors [12]. This difference in pathogenesis between ovarian and peritoneal endometriosis might explain the difference between the methylation profiles of both type of endometriosis described here.
5. Conclusion
Our results further support the theory of hypomethylation in the SF-1 gene promoter in endometriosis, and especially peritoneal endometriosis. The statistically significant difference between the SF-1 promoter methylation profiles in ovarian and peritoneal endometriosis might suggest differences in pathogenesis of both types of endometriosis. Since we obtained contradicting results with previous studies in endometriosis, more studies, especially regarding ovarian endometriosis, should be conducted. Further studies regarding expression of protein SF-1 and other gene in estrogen biosynthesis pathway in endometriosis could help to better determined the pathogenesis of peritoneal and ovarian endometriosis.

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