DNA methylation of the progesterone receptor B (PR-B) gene promoter in human eutopic endometrium, ectopic peritoneum, and ovarian endometriosis

Darmawi¹, M L S Marwali², R R Febri³, R Muharam³,4 A Hestiantoro³,4 and Asmarinah³,5*

¹Master Program in Biomedical Sciences, Faculty of Medicine, Universitas Indonesia, Jakarta 10430, Indonesia
²Endometriosis Center, Fatmawati General Hospital, Jakarta 12430, Indonesia
³Cluster of Human Reproductive, Infertility and Family Planning, Indonesian Medical Education and Research Institute, Faculty of Medicine, Universitas Indonesia, Jakarta 10430, Indonesia
⁴Division of Reproductive Immunoendocrinology, Department of Obstetrics and Gynecology, Faculty of Medicine, Universitas Indonesia /Cipto Mangunkusumo General Hospital, Jakarta 10430, Indonesia
⁵Department of Medical Biology, Faculty of Medicine, Universitas Indonesia, Jakarta 10430, Indonesia

*E-mail: asmarinah.si@gmail.com

Abstract. Endometriosis, a chronic inflammatory disorder, is characterized by the presence of hormone-responsive, endometrial-like tissue outside of the uterine inner wall, such as in the peritoneum and ovaries. Progesterone (P) resistance, due to altered expression of progesterone receptor (PR), is known as a disruptive condition in response to P in eutopic endometrial tissue. Gene promoter DNA methylation, a gene silencing mechanism, has been associated with the etiology of endometriosis. The aim of this cross sectional study was to assess the DNA methylation status of the PR-B promoter in various tissues (eutopic endometrium, ectopic peritoneal, and ovarian lesions) from endometriosis patient. This study involved 20 samples for each tissue type (eutopic endometrium, peritoneal, and ovarian lesion from women with endometriosis), as compared with 20 eutopic microcurettage samplings of normal endometrial tissue. DNA was isolated from each sample and subjected to bisulfite conversion. The DNA methylation level of the PR-B gene promoter was analyzed by the methylation-specific PCR (MSP) method. Band intensities in agarose gels were measured with ImageJ software. The ratio of the band intensity of samples to that of the positive control was considered as the DNA methylation level. The Mann–Whitney U test and Wilcoxon test were conducted, and P-values were considered significant at < 0.05. There were significant differences in the methylation levels of the PR-B gene promoters in ectopic peritoneal endometrial tissue (72.40% methylated), ovarian tissue (85% methylated), and eutopic endometrial tissue (72.21% methylated), as compared to normal endometrium (P = 0.000). Moreover, there were no significant differences in methylation levels of the endometriosis samples, i.e., peritoneal vs. eutopic endometrial tissue, peritoneal vs. ovarian endometrial tissue, and ovarian vs. eutopic endometrial tissue (P = 0.636, 0.241, and 0.441, respectively). Hypermethylation of the PR-B gene promoter could cause P resistance in different types of endometriosis lesions and might be a potential biomarker for diagnosis of endometriosis.
1. Introduction

Endometriosis is a chronic condition characterized by the presence of hormone-responsive, endometrial-like tissue outside of the uterine cavity that causes pelvic pain and infertility, and is mostly found in the peritoneal space, ovaries, and rectovaginal septum, and rarely in the pleura, diaphragm, and pericardium [1-3]. Endometriosis affects 6%–10% of women of reproductive age and up to 50% of infertile women. This variation is due to the diagnostic criteria of various epidemiological studies [4]. For example, population-based studies conducted in Germany (2012) and Israel (2017) reported that the prevalence of endometriosis was 8.1 and 12.8 cases per 1000 women of reproductive age, respectively [5,6]. Clinically, endometriosis can involve any human organ and develops from the onset of menarche to menopause. Depending on the location of the lesion, up to 20% women with endometriosis are infertile. The most common symptoms of endometriosis are chronic pelvic pain (40%–50%), dysmenorrhea (58%–80%), dyspareunia (40%–50%), dysuria (1%–2%), dyschezia (1%–2%), gastrointestinal discomfort (1%–2%), and decreased libido [2]. Pelvic pain during the menstrual cycle is caused by neurogenic inflammation and increased permeability of blood vessels due to constriction and infiltration of the endometriotic lesion to the surrounding nerves [7]. Infertility in endometriosis is due to decreased egg and embryo quality, and unsuccessful implantation [8].

Estrogen (E) overproduction and progesterone (P) resistance are believed to be aggravating factors in the clinical manifestations of endometriosis. An increase in E levels in endometriotic tissue is due to a high level of aromatase enzyme, inactivation of 17-beta hydroxysteroid dehydrogenase type 22, overexpression of steroidogenic factor 1, and hypomethylation of the promoter of estrogen receptor beta (ER-β) [9]. P resistance is reportedly due to low expression of the progesterone receptor (PR) in both ectopic and eutopic endometrial tissue [10]. The binding of P to the PR causes suppression of ER-β in the E-primed endometrium. There are two PR isoforms: PR-A and PR-B. PR-B functions as an activator of many transcription factors, whereas PR-A acts as a suppressor [10]. Moreover, PR-A has been described as a transrepressor of PR-B in ectopic endometrial tissue rather than the eutopic counterpart [2].

Available data suggest that methylation of the promoter and expression of PR-B could be used as biomarkers for the early detection and diagnosis of endometriosis. The ability of endometrial cells to implant and grow at different locations outside of the uterus, such as the ovaries, peritoneum, rectovaginal septum, and cul-de-sac of Douglas, suggests difference in the pathogenesis of P resistance. Hence, the aim of this study was to assess the methylation status of the PR-B gene promoter at various locations outside of the uterus, including the eutopic endometrial tissue, peritoneum, and ovarian ectopic endometrial tissue. The findings of this study will help to further elucidate the etiology and pathomechanism of endometriosis for diagnostic, prognostic, and even therapeutic uses.

2. Materials and Methods

The study protocol was approved by the Ethics Committee of the Faculty of Medicine of Universitas Indonesia and Cipto Mangunkusumo Hospital (approval no. 464/UN2.F1/ETIK/2017). This cross sectional study involved 20 eutopic endometrial, peritoneal, and ovarian lesions from endometriosis patient aged < 45 years, and 20 eutopic microcurettage samplings of the endometrium of endometriosis-free infertile women aged < 45 years. All samples were obtained laparoscopically or laparotomically and histopathologically confirmed. Sample collection was conducted according to the protocol of the World Endometriosis Research Foundation [11]. Endometriotic and eutopic endometrial tissues were collected during the proliferative menstrual phase, while control samples of normal endometrium were collected during the luteal menstruation phase.

About 30 mg of each tissue sample were homogenized in a 1.5-mL microcentrifuge tube. DNA was extracted using the gSYNCTM DNA Extraction kit (Geneaid, New Taipei City, Taiwan) Then, about 1500 ng of isolated DNA was used for bisulfite conversion with the Epitect Bisulfite kit (Qiagen, Hilden, Germany). Methylation-specific polymerase chain reaction (MSP) was used to assess the
methylation status of the PR-B promoter (Epitect MSP kit, Qiagen). The web-based programs http://www.cbs.dtu.dk/services/Promoter and http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi were used to identify the promoter regions and design methylation-specific primers, respectively. There were two CpG islands in PR-B promoter and the methylation-specific primers were (F) 5'-TGT TAT TAA ATT GTC GTC GTA GTC G-3' and (R) 5'- TAA AAA ATC TCG TCT CCT AAG TCG A-3'. The non-methylation-specific primers were (F) 5'- TGT TAT TAA ATT GTT GTT GTA GTT GTT 3' and (R) 5'- AAA AAA TCT CAT CTA ACT CAA A-3'. The final amplification products were 200-bp in length with an annealing temperature of 47.5°C. MSP was also applied to the positive control samples (EpiTect Control DNA (human), methylated and bisulfite converted, Qiagen).

The MSP amplification products were confirmed by electrophoresis with 1.5% agarose gels. ImageJ software (https://imagej.nih.gov/ij/index.html) was used to measure band intensity. The ratio of the band intensity of samples to that of the positive control was considered as the DNA methylation level. Data of paired patient samples were analyzed using the Mann–Whitney U test and Wilcoxon test. All data analyses were conducted using IBM SPSS Statistics for Windows, version 23.0 (IBM Corporation, Armonk, NY, USA).

3. Results and Discussion
The PR-B promoter methylation levels of the peritoneal, ovarian ectopic, and eutopic endometrium of endometriosis patients were 72.40%, 85%, and 72.21%, respectively, whereas the control normal endometrium was 100% unmethylated. The band intensities of eutopic endometrium, ectopic peritoneal, and ovarian endometrial tissues, as well as the positive control and normal endometrial tissues are shown in Figure 1.

Two independent samples of each endometriosis group were compared with the control normal endometrium. The results of the non-parametric Mann–Whitney U test revealed significant differences in PR-B promoter methylation levels between the peritoneal ectopic endometrial tissue, ovarian ectopic endometrial tissue, and eutopic endometrial tissue vs. the control tissues (p = 0.000). In addition, there were no significant differences in the Wilcoxon test results among the various tissue samples of paired endometriosis patients. Lastly there were no significant differences in the PR-B promoter methylation levels among the peritoneal ectopic and eutopic endometrial tissues, the peritoneal and ovarian ectopic endometrial tissues, or the ovarian ectopic and eutopic endometrial tissues (p = 0.636, 0.241, and 0.441, respectively).

![Figure 1](image_url). Electrophoresis of MSP products with the PR-B methylated (M) and unmethylated (U) primers. Peritoneal (P6 dan P7), ovarian (O7), ectopic lesion, and eutopic endometrial (U7) tissues of endometriosis patients compared with the control normal endometrial tissue (K20). M; DNA marker, C (+); positive control, NTC; non-template control.
Figure 2. PR-B gene promoter methylation levels of the eutopic endometrium, ectopic ovarian, and peritoneal endometrial tissues as compared to normal endometrium.

4. Discussion
Alterations in DNA methylation are highly associated with gene silencing and genome instability [12]. In endometriosis, which is likely a carcinogenic disease, the CpG islands of various gene promoters undergo hyper- and hypomethylation [13]. Wu et al. reported that the promoters of the PR-B gene were hypermethylated in ectopic endometrial tissues of 12 endometriosis patients, as compared with four control samples [14]. Furthermore, in endometriotic ovarian tissues of 20 patients, Febri et al. reported that the mRNA expression levels of PR-A and PR-B were decreased, and the expression of the hypermethylated PR-B gene promoter was lower than that of the controls. This result is consistent with the results of the present study that the promoter was hypermethylated in three kinds of endometrial tissues.

Although there were no significant differences in DNA methylation levels of various endometrial tissue samples, DNA methylation was more extensive in the ovarian ectopic endometriotic lesions as compared the eutopic endometrium and ectopic peritoneal endometriotic lesions. This condition could decrease PR levels resulting in P resistance in the ovaries.

Decreased PR expression and subsequent P resistance have been reported in several studies. For example, Attia et al. reported decreased PR-B expression in endometriotic tissue at both the mRNA and protein levels. A microarray study of endometrial cells collected from endometriosis patient also reported decreased expression of various downstream P-regulated genes, such as glycodelin, N-acetylglucosamine-6-O-sulfotransferase, and 17β-hydroxysteroid dehydrogenase 215-17. In addition, Xue et al. suggested that the hypomethylation level of the promoters of steroidogenic factor 1 and ER-β in endometriotic cells leads to E overproduction, decreased estradiol conversion to estrone, and increased P resistance [8].

In order to develop a biomarker of the DNA methylation status of various genes, a specific location within the genome must be chosen carefully due to the coincidence of both hypermethylated and demethylation of the CpG islands in the same disease. Besides, such biomarkers would be useful to elucidate the pathomechanisms of disease. Epigenetic biomarkers allow for the early diagnosis of disease and could be used as prognostic tools to assess progression and therapeutic responses [12].

5. Conclusion
There were no differences in the methylation levels of the PR-B gene promoters in eutopic and ectopic endometrial tissues. Thus, DNA methylation might be useful as a potential biomarker of
endometriosis. These findings could be implemented for further discovery of diagnostic and prognostic biomarkers.

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