Promoter hypermethylation in progesterone receptor-A (PGR-A) and PGR-B gene decreased its mRNA expression in ovarian endometriosis

Ririn Rahmala Febri[1], R Muharam[1,2], Asmarinah[3]*

1. Human Reproductive, Infertility and Family Planning Research Center, Indonesian Medical Education and Research Institute, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia.
2. Department of Obstetrics and Gynecology, dr. Cipto Mangunkusumo General Hospital, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia.
3. Department of Medical Biology, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia.

Abstract

The pathophysiology of endometriosis is not fully understood. It is believed that Progesterone Receptors (PGRs) play an important rule in the development of the disease. PGRs are expressed in human endometrial stromal. Reduction level of PGRs is related to the molecular basis of progesterone resistance in endometriosis. The aim of this study was to evaluate the association between methylation level of PGR-A and PGR-B gene and its mRNA expression in ovarian endometriosis.

We used endometriotic tissues from 20 patients, aged 20-35 years, compared to endometrial tissues obtained from 20 normal women, age-matched to endometriosis group, who underwent micro-curettage to evaluate methylation status and mRNA expression of PGR-A and PGR-B gene. Methylation status of both PGRs was analyzed using MSP, and mRNA expression was investigated using qPCR.

Methylation level of PGR-A (10,84%) and PGR-B (98,72%) in ovarian endometriosis were significantly different compared to control. The mRNA expression of PGR-A and PGR-B in ovarian endometriotic tissues significantly decreased by 2.35 and 6.37 folds compared to the normal endometrial tissues. Hypermethylation of PGR-B gene was correlated to mRNA expression, while methylation level of PGR-A had no correlation to mRNA expression.

Promoter regions of PGR-A and -B genes in ovarian endometriosis are hypermethylated compared to controls. These hypermethylationmay reduce their expressions, hence might contribute to pathogenesis of endometriosis.

Clinical article (J Int Dent Med Res 2019; 12(1):242-246)

Keywords: DNA methylation, Endometriosis, mRNA, PGR

Received date: 20 August 2018 **Accept date:** 25 September 2018

Introduction

Endometriosis is a chronic gynecological disorder, manifested by the presence of endometrium-like tissue that grows and develops outside the uterus, affects 10-15% of all women of reproductive age and 70% women with chronic pelvic pain.[1] The prevalence of endometriosis in women varies greatly, in Asian women is 15.7% which is greater than Caucasian women (5.8%).

Corresponding author:
Asmarinah
Department of Medical Biology,
Faculty of Medicine, Universitas Indonesia,
Jakarta, Indonesia.
E-mail: asmarinah.si@gmail.com

Progesterone induces the differentiation of epithelial and stromal cells, decrease in mitotic figures and cell proliferation.[1] Progesterone resistance has been found in endometriosis and associated with the low level of its receptor.[2] Progesterone and its receptor (PGR) control the proliferation of endometrial wall.[3,4] Extremely low PGR expression causes progesterone resistance and predicted then cause endometriosis.[5,2] Progesterone receptor has two isoforms, PGR-A and PGR-B, which are transcribed from the single gene.[6] Low receptor expression is often associated with hypermethylation in the promoter region of the gene.[7] However, the mechanism responsible for decreased PGR expression in ovarian endometriosis is still under investigation. Most of what is known about the roles of each receptor in endometriosis is derived from studies

of peritoneal and ovarian lesions. Wu et al reported that the promoter region of PGR-B is hypermethylated in endometriosis as compared with controls, while PGR-A is not.[8] In this study, we evaluated the methylation status of PGR-A and –B gene promoter regions in ovarian endometriosis tissue as well as its mRNA expression compared to normal endometrial tissues.

Materials and methods

Sample collection

Endometriotic tissues were taken from 20 patients, with surgically and histologically confirmed stages III-IV ovarian endometriosis. The patients, age ranged from 20 to 35 years and were not taking any medications prior to surgery. The exclusion criteria for endometriosis group were endometrium cancer, ovarian cancer and endometritis. Control samples of endometrial biopsies were obtained from 20 healthy women, aged 20-35 years, who underwent micro-curettage prior to assisted-reproductive technology and laparoscopically confirmed to be free of endometriosis. The information on the phase of menstrual cycle at the time of tissue collecting was determined according to the criteria of Noyes et al.[9] The DNA from tissue samples was extracted using Wizard® Genomic Purification Kit (Promega A1125, USA).

Bisulfite conversion

Treatment of genomic DNA with sodium bisulfite can deaminate unmethylated cytosine residues to uracil under conditions, while 5-methylcytosine is deaminated at a very slow rate.[10] The bisulfite conversion process used Epitect Bisulfite Kit (Qiagen 59104, Germany).

Methylation Specific Polymerase Chain Reaction (MSP)

The MSP process used KAPA Hifi Hotstat Uracil + Ready mix PCR (KAPABiosystems KR413-v2.13, Massachusetts, US); and two primers those are methylated and unmethylated. The PCR primers are listed in Table 1. The MSP product and Epitect® human methylated DNA control were plotted in agarose gel. Throughout the MSP analysis; a methylated (M) band appeared when the CpG sites were methylated, an unmethylated (U) band while unmethylated, and both bands appeared while they partially methylated. Methylation level was determined by the intensity of the bands between the samples and controls, analyzed by ImageJ software.

RNA extraction

Total RNA was extracted using High Pure RNA Isolation Tissue Kit (Roche, USA), taken from the specimens of patients and controls. Total RNA (10 ng) was reverse-transcribed (Transcriptor First Strand cDNA Synthesis Kit, Roche, USA), then the mRNA expression of PGR-A and PGR-B were further quantified by a real time PCR analysis, using Universal Probe Library and Light Cycler Taqman Probe Master Mix (Roche, USA). The primer sets for qPCR are listed in Table 2. The amount of mRNA level of PGR from each subject was normalized with β-actin ACTB mRNA as endogenous control, taken from the same subject. Relative expression of PGR-A and PGR-B mRNA was determined using Livak's method ($2^{-\Delta\Delta Ct}$) by comparing the relative differences between the cyclic threshold (Ct) of the genes in ovarian endometriosis and normal endometrium, after normalized with ACTB.[11]

All procedures of this study were approved by the responsible committee on human experimentation from Ethics Committee of Faculty of Medicine Universitas Indonesia. No: 380/UN2.F1/ETIK/2016 and with the Helsinki Declaration of 1964 and its later amendments. Informed consent was obtained from all patients and controls for being included in the study.

Data were analyzed using the Statistical Package for the Social Sciences version 22.0 software (SPSS Inc., Chicago, IL, United States). We used Shapiro-Wilks test to assess normality of distribution of continuous variables. Normally distributed data was presented in mean±SD, while not normally distributed was presented in median (interquartile range). We used independent T-test to determine the difference of methylation level of PGR-A and PGR-B between two groups, as well as mRNA expression of these receptors. Spearman's rho test, correlation between normally and non-normally distributed variables, was used to analyze the correlation between methylation level and mRNA expression. Furthermore, two-tailed p value < 0.05 was considered significant.

Results

Forty women were recruited to participate in this study and were further classified into 2 groups according to the presence or absence of endometriosis. In overall, age of women of group endometriosis and control were not statistically different (age: 35.25 ± 4.65 and 36.13 ± 4.65 years, respectively, p>0.05). The MSP products were visualized using gel electrophoreses as a single band (Fig. 1). The intensity of the PGR-A and PGR-B methylation bands was found higher among the ovarian endometriosis, around 10.84% and 98.72%, respectively, compared to control. Independent T-test was performed to determine the different of methylation levels among two groups. There was a significant difference in the intensity of the methylation level of PGR-A and PGR-B bands between the endometriosis patients to the control (p<0.05) (Fig. 2).

The level of PGR-A and PGR-B mRNA expressions were significantly different in patients with endometriosis (p<0.05), which decreased by 2.35 fold (0.426±0.024) and 6.37 fold (0,157±0,011) in endometriosis group compared to the control group (Fig. 3). There was a significant correlation between methylation levels of PGR-B to its mRNA expression in endometriosis group (p<0.001; r=-0.736). However, no correlation was found between PGR-A methylation to the mRNA expression (p>0.05; r=-0.387). Correlation graphs were presented in Figure 4.

Discussion

In this study, the expression of PGR-A and PGR-B was significantly decreased in ovarian endometriosis compared to normal endometrium. Bulun et al (2006), who investigated the PGR transcripts in endometriosis using RNase protection assay, showed that PGR-B were absent in endometriotic tissue samples and very low levels of PGR-A, whereas both PGR-A and PGR-B transcripts were detectable in all normal endometrium samples.[1]

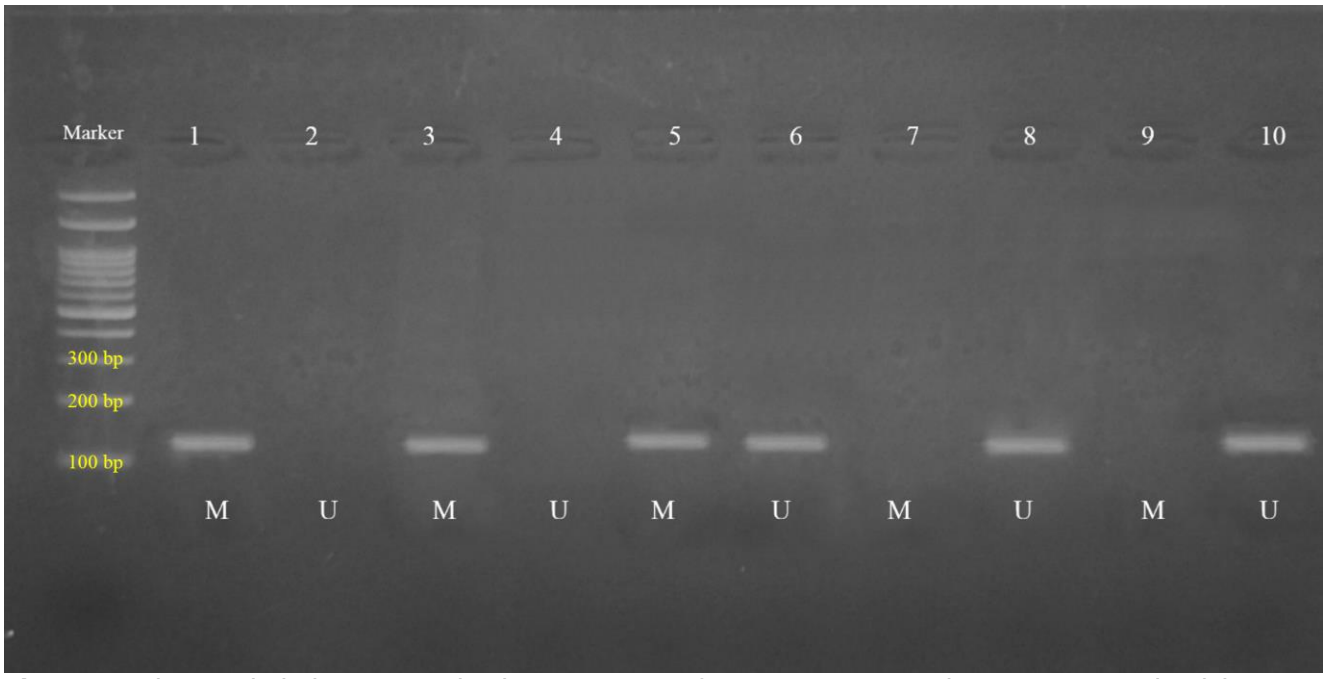


Figure 1 The methylation status in the promoter of PGRs. PCR products were resolved in 2.8% agarose gel. These primers contain 7 CpG sites where 35% of ovarian endometriosis samples are partially methylated (line 5 and 6) shown by methylated and unmethylated band, 65% of ovarian endometriosis samples are completely methylated (line 3) and only 10% normal endometrial samples had methylated band (lanes 7-10). (1): methylated control. (2): PCR negative control. M: methylated and U: unmethylated.

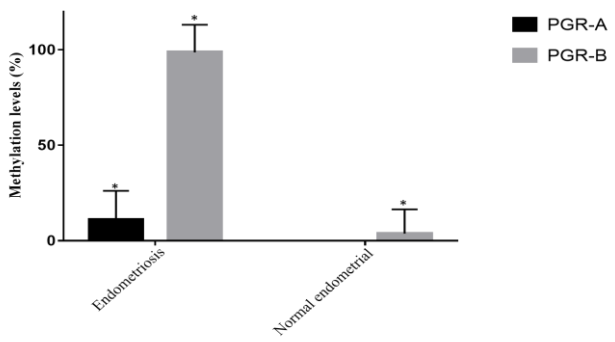
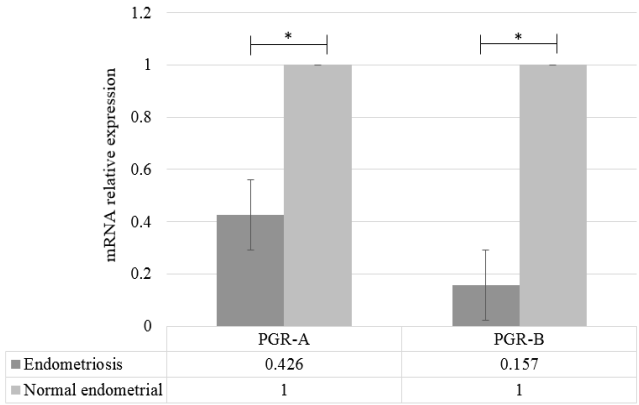
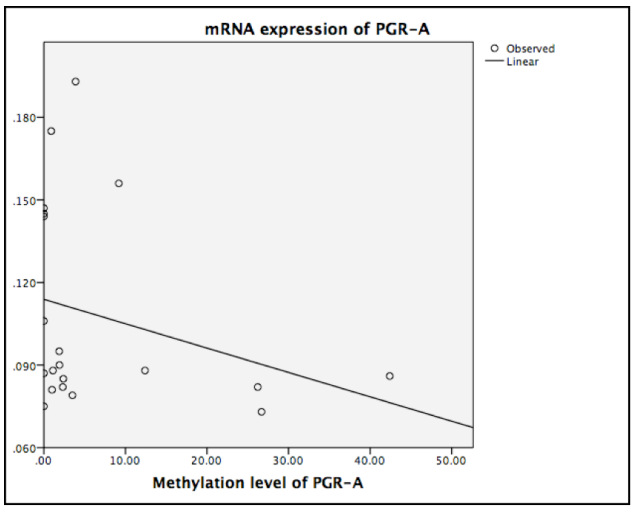


Figure 2 The difference of methylation status between ovarian endometriosis tissues with normal endometrium from micro-curettage patients. The percentage of methylation status from each sample was analyzed using ImageJ compared to positive control. Data are expressed in mean ± SD.

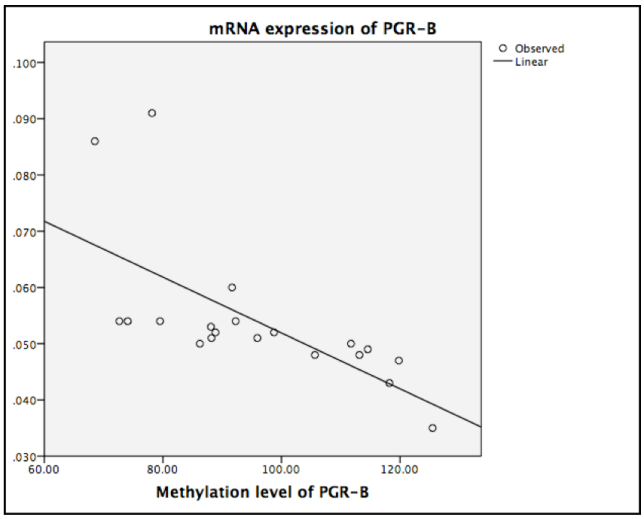


	PGR-A	PGR-B
Endometriosis	0.426	0.157
Normal endometrial	1	1

Figure 3 Relative expression of PGR-A and PGR-B mRNA in endometriosis tissue. The y-axis represents fold change in expression as determined by quantitative real-rime PCR and is expressed as mean±SEM. The x-axis represents the measured groups.



a



b

Figure 4 Correlation graphs between methylation level of PGRs and its mRNA expression in ovarian endometriosis. (a) PGR-A; (b) PGR-B. There was negative correlation between DNA methylation in PGR-B gene and its mRNA expression significantly ($p<0.001$; $r=-0.736$) but not significant for PGR-A gene ($p>0.05$; $r=-0.387$)

On the other hand, Smuc et al (2009) demonstrated that no significantly difference in transcript expressions of PGR-A and -B in endometriosis, compared to controls (p>0,05).[12] Moreover, our study showed that the promoter of PGR-A and PGR-B were hypermethylated in ovarian endometriosis compared to control. Similar to our study, Wu et al (2006) using MSP showed hypermethylation of PGR-B promoter in endometriosis specimens that taken from any area, compared to control group; so did Meyer et al (2014) who found hypermethylated promoter of PGR that occurs among endometriosis, while the expressions were still detected among patients with endometriotic lesions, compared to normal endometrium obtained from premenopausal patients.[8,13] However, methylation level of PGR-A in these studies was unexplored. DNA methylation, as one of epigenetic mechanism, plays an important role in controlling gene transcription, genomic stability, embryonic development, gene silencing and gene regulation

cycle.[14] It has been known that promoter hypermethylation is associated with transcriptional silencing.[8] Our result suggested that hypermethylation of PGR gene, especially PGR-B had led to a low level of PGR-B mRNA expression in ovarian endometriosis, up to more than 6 times lower than its expression in normal endometrium.

Here are we explained about the limitations of our study, including small sample size, cross-sectional and experimental design with no follow-up, and inability to generalize the results to other populations.

Conclusions

In conclusion, we found a negative correlation between hypermethylation and mRNA expression of PGRs in endometriosis. Our results suggested that DNA hypermethylation might contribute to the down regulation of progesterone receptor expression in the ovarian endometriosis and may plays a major epigenetic mechanism of functional progesterone withdrawal at the pathogenesis of endometriosis.

Acknowledgment

We gratefully acknowledge Achmad Kemal Harzif and Catherine for their technical support. The publication of this manuscript is supported by Universitas Indonesia.

Declaration of Interest

We declare that we have no conflict of interest.

References

1. Bulun SE, Cheng YH, Yin P, et al. Progesterone Resistance in Endometriosis: Link to Failure to Metabolize Estradiol. Mol Cell Endocrinol 2006;248(1-2):94–103.
2. Kim JJ, Kurita T, Bulun SE. Progesterone Action in Endometrial Cancer, Endometriosis, Uterine Fibroids, and Breast Cancer. Endocr Rev 2013;34(1):130–62.
3. Kim JJ, Chapman-Davis E. Role of Progesterone in Endometrial Cancer. Semin Reprod Med 2010;28(1):81–90.
4. Takai E, Taniguchi F, Nakamura K, Uegaki T, Iwabe T, Harada T. Parthenolide Reduces Cell Proliferation and Prostaglandin Estradiol Synthesis in Human Endometriotic Stromal Cells and Inhibits Development of Endometriosis in the Murine Model. Fertil Steril 2013;100(4):1170–8.
5. Nasu K, Kawano Y, Tsukamoto Y et al. Aberrant DNA Methylation Status of Endometriosis: Epigenetics as the Pathogenesis, Biomarker and Therapeutic Target. J Obstet Gynaecol Res 2011;37(7):683–95.
6. Kaya HS, Hantak AM, Stubbs LJ, Taylor RN, Bagchi IC, Bagchi MK. Roles of Progesterone Receptor A And B Isoforms During Human Endometrial Decidualization. Mol Endocrinol 2015;29(6):882–95.
7. Bulun SE, Cheng YH, Pavone ME, et al. Role of Estrogen Receptor-B, Estrogen Receptor-A, and Progesterone Resistance in Endometriosis. Semin Reprod Med 2010;28(1):36–43.
8. Wu Y, Strawn E, Basir Z, Halverson G, Guo SW. Promoter Hypermethylation of Progesterone Receptor Isoform B (PR-B) in Endometriosis. Epigenetics 2006;1(2):106–11.
9. Noyes RW, Hertig AT, Rock J. Dating The Endometrial Biopsy. Am J Obstet Gynecol 1975;122(2):262-3.
10. Dahl C, Guldberg P. DNA Methylation Analysis Techniques. Biogerontology 2003;4(4):233–50.
11. Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2(-Delta Delta (CT)) Method. Methods 2001;25(4):402–8.
12. Smuc T, Hevir N, Ribic-Pucelj M, Husen B, Thole H, Rizner TL Disturbed Estrogen and Progesterone Action in Ovarian Endometriosis. Mol Cell Endocrinol 2009;301(1-2):59–64.
13. Meyer JL, Zimbardi D, Podgaec S, Amorim RL, Abrão MS, Rainho CA. DNA Methylation Patterns of Steroid Receptor Genes ESR1, ESR2 and PGR in Deep Endometriosis Compromising The Rectum. Int J Mol Med 2014;33(4):897–904.
14. Koukoura O, Sifakis S, Spandidos DA. DNA Methylation in Endometriosis (Review). Mol Med Rep 2016;13(4):2939–48.