DNA methylation of P2X3 receptor gene encoded pain marker protein in endometriosis

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Abstract. Endometriosis is a common, benign, oestrogen-dependent, chronic gynaecological disorder associated with pelvic pain and infertility. Increasing of P2X3 receptors induced sensitization of pain in endometriosis patients. Epigenetic mechanism such as DNA methylation could lead to alteration of gene expression. The aim of this study was to analyse DNA methylation of P2X3 receptor gene promoter in peritoneal endometriotic tissue from 9 patients compared to endometrial tissue from 9 without endometriosis women as control. The DNA from samples was isolated and with sodium bisulfite converted. We used Methyl Specific PCR (MSP) method to amplify the DNA and then running MSP product in gel electrophoresis. The band intensity of samples were measured by ImageJ software. Statistical analysis was significant correlation between pain and endometriosis (p= 0.000). DNA methylation of P2X3 receptor gene promoter among peritoneal endometriotic tissue in women endometriosis and endometrial tissue woman without endometriosis were 100% unmethylated and there was no significant differences (p=0.287), although density of band unmethylated peritoneum endometriosis group was higher than control group. This study was suggesting that DNA methylation of P2X3 receptor gene promoter might be a potential biomarker to early diagnostic of endometriosis without invasive procedure in endometriosis patienst especially with pain symptoms.

1. Introduction
Endometriosis is defined as the presence of endometrial glands and stromal implantation outside the uterus, generally in the peritoneal, ovary and retrovaginal septum [1]. The incidence of endometriosis was reported that occurs in 6-10% of women in the general population and 35-50% for pain and infertility [2]. Based on the data of Cipto Mangunkusumo Hospital (RSCM) in Jakarta Indonesia as a national hospital, incidence of endometriosis was 13.6-69.5% in the infertile female group or...
approximately there were 2-8 million reproductive women in Indonesia that suffered endometriosis and more than 80% with pain symptom [3,4].

The recent study was suggest that epigenetics is one of the endometriosis pathogenesis [5,6]. Epigenetics is contributed to the difference in the risk of chronic pain. Research conducted on identical twins and showed that there was a difference in the response of chronic pain after adulthood; this might be influenced by nutrition, stress, or nerve injury [7].

Pain is one of symptoms in endometriosis, but how is the mechanism, it remains unclear. Recent evidence from several clinical studies suggest that endometriosis lesions was infiltrated by nerve fibers; nerve fibers density in lesions perhaps associated with endometriosis pain [8,9].

Bioinformatics-applied of microarray analysis suggests focal adhesion, regulation of actin cytoskeleton, the mitogen-activated protein kinase (MAPK) and transforming growth factor, beta 1 (TGFβ/SMAD) signaling pathway may be important molecular mechanism underlying the pathogenesis of endometriosis. This study was founded a total of 2255 up- and 408 down-regulated genes were identified in endometriosis patients as compared with control patients [10].

Cascade activation to increase the expression of purinergic receptor P2X ligand-gated ion channel 3 (P2X3) receptors gene occurred in pain signal transduction in endometriosis lesions. It was mediated by extracellular regulated protein kinase (ERK) via the Mitogen-Activated Protein Kinase (MAPK) signalling pathway. Increasing of P2X3 receptors induced sensitization of pain in endometriosis patients [11]. P2X3 was an appropriated P2X ligand channel subtype in the receptor member, which wasn't only neuronal but also non-neuronal in the receptor member like endometrium [12-14].

However, there is no study yet to analyzed DNA methylation of P2X3 receptors gene as a marker protein of pain in endometriosis patients and woman without endometriosis. The purpose of this study was to measure DNA methylation P2X3 receptors gene promoter in peritoneal endometriotic tissues of endometriosis woman and endometrial tissue of woman without endometriosis.

2. Methods
The endometriosis samples were collected after all participants signed an informed consent form. This study used 2 kinds of tissue, i.e. peritoneal endometriotic tissue and normal endometrial tissue as a control. The samples of peritoneal endometriotic tissue were collected from nine patients in the age of 20-45 years old, who came to Fatmawati Hospital, diagnosed by histopathology and collected by laparascopy. The samples of eutopic endometrial tissues were collected using microcuretase from nine women without endometriosis. The pain sensation was documented using a standardized questionnaire with a visual analog scale (VAS). We used three kinds of category pain scale that were no pain (VAS=0-1), mid pain (3-7), severe pain (8-10). The DNA from samples was extracted using Wizard® Genomic Purification Kit (Promega A1125, USA), quantified using the Maestrogen Maestro Nano Spectrophotometer (USA) and then converted by sodium bisulfite using an Epitect Bisulfite Kit, Qiagen 59140. For DNA methylation profiling, the bisulphite–converted DNA 20 ng was amplified by the methylation-specific polymerase chain reaction (MSP) using KAPA Hifi Hotstart Uracil+ReadyMix kit (KAPA Biosystem KR413-v2.13, Massachussets US).

Methylated and unmethylated primers for MSP method were designed using MethPrimer program. The methylation-specific primer were (F) 5’-GGG AGTTATGGTAACGAGTCGC-3’ and (R) 5’-GCCAAATATAAAAAACGATACGA-3’. The unmethylation-specific primer was (F)5’GGAGTTATGGTAATGAGGTGG-3’ and (R)5’-CCACAAATAATAAAAACATACAA-3’. The final amplification product was 120 in length with an annealing temperature of 49 °C.

MSP product was visualized in gel electrophoresis using the ultra violet light. The band intensity of MSP product from patients and control samples as well as positive control were determined using ImageJ program. DNA methylation of P2X3 receptor gene was calculated as a ratio of intensity methylated or unmethylated band to total intensity of methylated and unmethylated band. If it comes only one band in methylated or unmethylated wells, the difference of band intensity was compared
between samples. The data was analysed statistically using by independent T-test and Mann-whitney U test that significances was considered at p value <0.05.

3. Results

This study was measured homogeneity of age among samples that there were no significant differences between women with and without endometriosis regard to age (p>0.05) and measured correlation between pain and endometriosis that there was associated between pain and endometriosis (p=0.000) (table 1).

Table 1. Characteristics of study sample between endometriosis patients and without endometriosis.

| Characteristic | Endometriosis (n=9) | Without endometriosis (n=9) | p^a |
|---------------|---------------------|----------------------------|-----|
| Age (years: mean±SD) | 36.33±5.36 | 32.44±3.94 | 0.99 |
| Pain | | | | |
| 1. No pain | 0 | 9 | 0.000^a |
| 2. Mid Pain | 5 | 0 | |
| 3. Severe pain | 4 | 0 | |

^a p<0.05

The gel electrophoresis of MSP product of P2X3 receptor gene in peritoneal endometriotic tissue from endometriosis women and endometrial tissue from without endometriosis (Figure 1.a and 1.b).

Figure 1a,b. Electrophoresis MSP product of P2X3 receptor promoter gene in nine samples of peritoneal endometriotic tissue (P) and nine samples of endometrial tissue from women without endometriosis (K). Lanes = Marker; M= methylated, U= unmethylated, K+M= positive control methylated, ntc = non template control.

This study showed that P2X3 receptors gene amplification in peritoneal endometriotic tissue and eutopic endometrial tissue in patients without endometriosis were 100% unmethylated (figure 1a,b). Lastly there were no significant differences DNA methylation of the P2X3 receptor gene promoter among peritoneal endometriotic tissue in woman endometriosis and endometrial tissue woman without endometriosis (p=0.287). The band intensity of unmethylated in peritoneal endometriotic tissues were higher than normal endometrial tissues (figure 2).

4. Discussion

Aberrant gene expression has been demonstrated repeatedly and consistently in endometriosis[15]. In human, DNA methylation is a crucial epigenetic modification of genome that plays an important role in the regulation of gene expression and genomic imprinting [16]. One of kind epigenetic mechanisms was played a role in the occurrence of endometriosis is methylation of deoxyribonucleic acid (DNA) [17,18].
Modification of epigenetic is potential to play an important role in the metabolism of inflammatory cytokines, steroid response, and sensitivity of opioids, they are likely a key factor in the development of chronic pain. In addition, the study was suggest a different risk of chronic pain in identical twins, showing chronic pain after adulthood can be affected by epigenetic factors, nutrition and stress, and nerve injury [7].

This study founded that DNA methylation of P2X3 receptor gene promoter in peritoneal endometriotic tissues was no difference significantly compared to eutopic endometrial tissue and that were 100% unmethylated. Although band intensity of unmethylated P2X3 receptor gene promoter in peritoneal endometriotic tissue was higher than eutopic endometrial tissue.

DNA methylation regulates gene expression by inhibiting of transcription factor to bind to the gene promoter and also altering the chromatin structure. If the CpG islands of the promoter region for a particular gene are methylated, the expression of that gene may be down regulated or transcriptionally silenced; in contrast, if the CpG islands in the promoter region remain unmethylated, the gene is typically more highly expressed [19,20].

This study was found there was correlation between pain and endometriosis (p=0.000). Contrast by the recent study that suggest women with surgically visualized endometriosis reported the highest chronic pain and significantly greater dyspareunia, dysmenorrhea, and dyschezia compared with women with other gynecologic pathology (including uterine fibroids, pelvic adhesions, benign ovarian cysts, neoplasms and congenital Müllerian anomalies) or a normal pelvis [21]. In addition, the endometriosis lesions was infiltrated by nerve fibers; nerve fibers density in lesions might be associated with endometriosis pain [8,9].

Pain mechanism in endometriosis via extracellular-regulated protein kinase (ERK) through Mitogen-Activated Protein Kinase (MAPK) pathway signalling was reported by increases P2X3 expression in endometriotic lesions [11].

Our results suggest DNA methylation of P2X3 receptors gene might play a key role in alteration of gene expression and regulated pain mechanism in endometriosis. The further studies we would be measure that correlation between genetic level of mRNA expression and DNA methylation level of P2X3 receptor gene promoter.

5. Conclusion
DNA methylation of P2X3 receptor gene promoter in peritoneum endometriotic tissue of patients with endometriosis compared to eutopic endometrial from patients without endometriosis was unmethylated. This study suggests that DNA methylation of P2X3 receptor gene promoter a potential biomarker to
early diagnostic of endometriosis without invasive procedure in endometriosis patients especially with pain symptoms.

6. References

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