Analysis of DNA methylation and its correlation with mRNA expression of epidermal growth factor receptor encoding for cytoskeleton regulating protein in peritoneal endometriosis tissue

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Abstract. It has been known that the EGFR have the role for regulation the cytoskeleton activity and its expression increased in endometriosis tissue. The aim of this study was to evaluate the DNA methylation of the EGFR gene that might cause the alteration of its mRNA expression in peritoneal endometriosis tissue. Samples were peritoneal endometriosis tissue from 20 endometriosis patients and 20 female of non-endometriosis patients. The DNA methylation of the EGFR gene was analyzed by the method of Methylation Specific PCR and ImageJ software, while its expression of mRNA was analyzed by the method of qRT-PCR. The DNA methylation in the EGFR gene in peritoneal endometriosis tissues increased compared to normal endometrial tissues (peritoneal endometriosis tissue = 56%, normal endometriosis tissue = 19%). The expression of mRNA EGFR gene in endometriosis peritoneal tissues was 1.341 fold increased relative to normal endometrium. There is no significant correlation between the DNA methylation with expression of mRNA EGFR (p = 0.947 and r = -0.016). Increasing of EGFR mRNA expression in endometriosis tissue that was not caused by alteration of its DNA methylation, have to play a role in the pathogenesis of endometriosis.

Keywords: Endometriosis, EGFR, DNA methylation, cytoskeleton
1. Introduction
Endometriosis is a common and complex disease characterized by the presence of endometrial-like tissue such as the gland or stroma, which is located outside the uterus, which occurs in reproductive women or premenopausal women with a prevalence rate of around 10% or 176 million in the world. However, the causes of endometriosis have not been known or found with certainty and are clear, because many factors can affect the development of endometriosis, including hormones, immune system, environmental, genetic and epigenetic factors [1]. Endometriosis peritoneum is a superficial lesion on the surface peritoneum [2]. In several studies that have been carried out, reported that mesothelial peritoneal cells that were damaged by exposure to menstrual blood from retrograde menstruation caused morphological changes in mesothelial cells from cuboid to columnar elongated resulting in stretching between cells [3-4]. And this change is related to the reorganization of the cytoskeleton because of the attachment or exposure of ectopic cells to the extracellular matrix (ECM) resulting in binding of ectopic endometrial cells in the peritoneal area [5].

In endometriosis, there are several genes that experience increased expression, one of which is the Epidermal Growth Factor Receptor (EGFR) gene. Increased expression of the EGFR gene can contribute to activating the Mitogen-activated Protein Kinase (MAPK) pathway in patients with endometriosis so that it is involved in the pathogenesis of endometriosis [6]. The activation stimulation of the EGFR gene via the ERK / MEK / MAPK signaling pathway can be used to activate several genes which promotes invasion of the tissue that causes degradation in ECM [7]. It is known that ECM has been shown to affect the cytoskeleton organization in cells of non muscle, cells of smooth muscle, and cells of striated muscle. Therefore, the occurrence of ECM degradation can affect the cytoskeleton so that it can change cell shape, cell motility, and cell function. Thus, EGFR is involved in the regulation of the cytoskeleton [6, 8].

In recent years, several biomolecular-based studies on endometriosis and epigenetic aberrations have proven that epigenetics is control of endometriosis and thus becomes one of the pathogenesis of endometriosis. This is evidenced by the presence of genes that undergo DNA methylation and their relationship to gene expression in endometriosis. However, even so, there are still genes that have not known the level of methylation, one of them is the EGFR gene, and the level of expression of the mRNA in the endometriosis peritoneal tissue and the correlation with the methylation of DNA is unknown.

2. Material and Methods
2.1. Sample collection
Samples were obtained from patients who came to the Immuno-endocrinology polyclinic of Department Obstetrics and Gynecology RSUPN Dr. Cipto Mangunkusumo Jakarta and Fatmawati Hospital Jakarta. Peritoneal endometriosis tissue samples obtained from women with endometriosis that was collected using laparoscopic or laparotomy techniques, whereas normal endometrial tissue samples obtained using microcuretase techniques from infertile women who do not suffer endometriosis and want to take the program to get offspring. Patients were informed of their consent before asking for their approval.

2.2. The DNA methylation analysis
The DNA from both samples were isolated by using gSYNC DNA Extraction Kit from Geneaid and then 30 ng of DNA conversion by bisulfite solution use the Epitect Bisulfite Kit form Qiagen Germany. Converted DNA then amplified by Methylated Specific Polymerase Chain Reaction (MSP) method with using methylated and unmethylated primer pairs for EGFR gene that have been previously designed. The MSP products are run in agarose gel and visualized using ultraviolet, and then results will appear like a band. The intensity of band the MSP product was analyzed by the ImageJ software. The percentage of methylation level was determined by ratio intensity of methylated band and unmethylated band.
2.3. The mRNA expression analysis.
Total RNA was isolated from both kinds of samples using Quick-RNA Miniprep Plus Kit from Zymo. In the amount of 50 ng of RNA was transcribed reverse to cDNA use Ace qPCR ReverTra RT Master Mix with gDNA Remover from Toyobo. The cDNA from both samples was then amplified using Thunderbird SYBR qPCR Mix with primer pairs for EGFR gene. The expression of mRNA EGFR gene in each subject was normalized using GAPDH mRNA which functions as an endogenous control. Analysis of the relative expression of the EGFR gene mRNA using the Livak (2^-∆∆Ct) method conducted by comparing the relative differences between the cyclic threshold (Ct) of the peritoneal endometriosis tissue and normal endometrial tissue.

2.4. Data analysis.
Data analysis used software of SPSS (Statistical Package for the Social Sciences). The test used is the Shapiro-Wilks test to determine the normality of the distribution of data. If normal distributed data is obtained, the data will be presented in mean + SD, whereas if the data is not normally distributed, the data will be presented in the median form. The independent t-test is a test used to determine the differences in expression of mRNA and the level of EGFR gene as well as DNA methylation level in endometriosis samples, and used the Pearson test to analyze the correlation between mRNA expression and DNA methylation of the EGFR gene in peritoneal endometriosis tissues. The p-value is considered significant if the value is p <0.05.

3. Results and Discussions
The DNA methylation of EGFR gene in the peritoneal endometriosis tissue 56% was increased compared to normal endometrial tissue 19%, and there is a significant differences between peritoneal endometriosis tissue with normal endometrial tissue (p = 0.001) (figure 1 and 2). The mRNA expression of EGFR gene in peritoneal endometriosis tissue was 1,346 fold relative higher than endometrial tissue (figure 3). However, the level of DNA methylation did not correlate with mRNA expression of EGFR. (p = 0.947) (figure 4)

Figure 1. The electrophoresis visualization of MSP product of EGFR gene. PET = Peritoneal Endometriosis Tissue; NET= Normal Endometrial Tissue; (+) = positive control; (-) = negative control; M = methylated band; U = unmethylated band.
Figure 2. DNA methylation of the EGFR gene. There is a significant difference of DNA methylation level of EGFR gene in peritoneal endometriosis tissue compared to normal endometrial tissue (*p = 0.001)

Figure 3. mRNA expression of the EGFR gene. In peritoneal endometriosis tissue an increased of 1.346 fold compared to normal endometrial tissue, and there is not significant difference (p = 0.235)

Pathogenesis of a disease in humans one of which is the occurrence of deviant or abnormal epigenetics. Endometriosis is a disease whose pathogenesis is due to deviant or abnormal epigenetics [1]. This study, we find the DNA methylation level of EGFR gene in peritoneal endometriosis tissue 56% higher than those in normal endometrium. Our result was supported by Montero et al that reported EGFR gene underwent hypermethylation in the primary solid tumors as well as in breast, lung, colorectal, head and neck cancer cell [9]. Likewise, a study conducted by Weng et al showed that the EGFR gene underwent hypermethylation in gastric cancer tissue [10]. Endometriosis is the presence and growth of tissue outside the uterus such as in the ovary, pelvic peritoneum, rectovaginal septum, etc, that are similar to endometrial tissue [11]. Another definition of endometriosis is a pathological disease caused by the proliferation of ectopic endometrial tissue in places other than the cavity endometrium. The pathogenesis of endometriosis has yet to be known with certainty and clarity, but there are already theories that explain or reveal the origin of endometriosis. The most widely supported theory is the theory discovered by a scientist named Sampson, the Theory of Retrograde Menstruation. Of the 90% of women who experience menstrual blood regurgitation during the menstrual cycle, there are only about 10% that cause endometriosis. Many factors that affect this occur, ranging from hormonal factors to developing epigenetic factors. Alteration in the epigenetic
mechanism could give effect in the regulation of gene expression. This study showed there was an increase in the relative expression of mRNA of EGFR gene in peritoneal endometriosis tissue to normal endometrial tissue. This study is in accordance with research conducted by Ping et al., which has found 2,255 genes increased expression in endometriosis tissue, one of them was EGFR gene. We found in this study, the increasing of mRNA expression of EGFR in peritoneal endometriosis tissue as much as 1.346 fold relative to normal endometrium tissue. It is known that the EGFR gene is a proto-oncogene [9,12]. Endometriosis is also a disease that has a malignant nature and can even become cancer, so this study is in accordance with previous studies that have been carried out on the EGFR gene in various types of malignancies. In a study conducted by Yanli Xu et al., It was shown that in endometrial carcinoma, EGFR was overexpressed [13]. In endometrial tumors, it was also shown to have excessive or high expression [14]. Weng et al in their study found that the EGFR gene increased expression in gastric cancer [10]. Increased EGFR expression is also found in breast, lung, colon, ovarian and brain tumors [15].

Figure 4. Correlation between of DNA methylation with expression of mRNA of the EGFR. There is no correlation significantly between of DNA methylation with expression of mRNA of the EGFR in peritoneal endometriosis tissue (p = 0.947 and r = -0.016).

Changes in expression that occur in a gene are caused due to genetic and epigenetic factors. In endometriosis, changes in the level of expression that occurs can be caused by epigenetic variations [16]. The expression of a gene is controlled by the presence of an epigenetic mechanism, namely DNA methylation so that the DNA methylation process can result in changes in gene expression. An increased in DNA methylation (hypermethylation) is usually associated with decreased gene expression, and decreased methylation (hypomethylation) is associated with high gene expression [17]. In this study, it was shown that there was no correlation between of the DNA methylation and the mRNA expression of EGFR in peritoneal endometriosis tissue.

This study is in line with the research conducted by Weng et al in gastric cancer showing that the EGFR gene has a positive correlation between mRNA expression with DNA methylation, which both increase of mRNA expression and DNA hypermethylation of EGFR gene [10]. However, in study conducted by Weng et al. found something different from usual, it is known that DNA hypermethylation usually will be causes silencing of genes in many situations, but this study of DNA hypermethylation causes an increase in mRNA expression. The biological basic and mechanism that allows hypermethylation of promoters which can cause an increase expression is still unknown. This
increase can occur probably because DNA methylation that occurs at the promoter region close to the initial transcription site leads to a three-dimensional change in chromatin conformation in this region, or this DNA methylation can prevent the binding of repressors which usually prevents gene expression in normal cells [10]. The increasing of gene expression may be caused by other factors, i.e. mutation in the promoter gene region and other epigenetic mechanisms such as histone modification in chromatin.

4. Conclusion
There is hypermethylation of the EGFR gene in peritoneal endometriosis tissue. Increasing of mRNA expression of EGFR in peritoneal endometriosis tissue that was not caused by alteration of its DNA methylation. Further research should be done to investigate the epigenetic mechanism that can intervene in the pathogenesis of endometriosis.

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