Analysis of mRNA expression and DNA methylation level of RAC1 gene encoding focal adhesion molecule in endometrial and peritoneal endometriosis

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Abstract. Focal adhesion molecules involve in cellular migration, attachment, and play a role in endometriosis pathomechanisms. Recent studies showed that the expression of RAC1, a gene encoded focal adhesion molecule, was predominantly found in endometriosis. As gene expression may be regulated by DNA methylation. Therefore, this study aimed to analyze promoter methylation level of RAC1 gene and mRNA expression in endometrial and peritoneal endometriosis tissues. This study using 20 endometrial and 9 peritoneal tissues from the same patients and 20 normal endometrial. The DNA and RNA from samples were isolated, DNA was converted using sodium bisulfite and amplified using Methyl Specific Polymerase Chain Reaction (MSP) method. The methylation level was determined by the intensity measurement of the bands that arose in gel electrophoresis using ImageJ software, whereas mRNA expression level was measured by Reverse Transcription-quantitative PCR (RT-qPCR) method. The mRNA expression level of RAC1 gene in peritoneal endometriosis increased compared to normal endometrium, as well as compared to endometrial endometriosis, but there was no significant difference in endometrial endometriosis compared to normal. Promoter hypermethylation level of RAC1 gene in peritoneal endometriosis was significantly different compared to normal endometrium, however not significant to endometrial endometriosis. Methylation level of its gene in endometrial endometriosis shown no significant difference compared to normal. There was association between promoter hypermethylation level and its mRNA expression in endometrial endometriosis ($R = 0.014$; $p = 0.952$). The elevation of mRNA expression of RAC1 gene plays a role in endometrial cell migration to peritoneum, and associated with promoter hypermethylation level of its gene.

Keywords: DNA methylation, focal adhesion, peritoneal, RAC1

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INTRODUCTION

Endometriosis often occurs in 6 to 10% women during their reproductive age. Endometriosis is defined as the presence of endometrial-like tissue located outside the uterine cavity that frequently found in ovaries and peritoneal (Sourial *et al.*, 2014; Barcz *et al.*, 2012). Common symptoms that may appear including dysmenorrhea, dyspareunia, and infertility. The widely accepted theory to explain endometriosis was Retrograde Menstruation containing cellular debris from menstrual endometrium and become a source for ectopic lesion (Soo *et al.*, 2017).

The development of lesion may be affected by immune systems, apoptosis resistance, growth factors, and adhesion molecules (Aznaurova *et al.*, 2014). Clusters of cell adhesion molecules such as integrins trigger cell attachment to a various extracellular matrix including fibronectin, laminin, and vitronectin drives cellular migration through interaction with the downstream regulation such as RAC1 (Burney *et al.*, 2012; Jiang *et al.*, 2012).

In recent years, extensive investigation of genes that may have contribution and association with endometriosis pathomechanisms has been conducted using genome-wide association studies (GWAS) (Liu and Zhao *et al.*, 2016). Ping *et al.* (2016) reported that RAC1 mRNA expression, a gene encoded one of focal adhesion molecules, was found increase predominantly in endometrium women with endometriosis. RAC1 is a member of the Rho GTPase family, which includes RHO, RAC1, and CDC42 (Bid *et al.*, 2013).

It has been known that focal adhesions act as a bridge between the cells and the extracellular matrix through integrins as a transmembrane protein that initiate adhesion and regulate assembly of actin cytoskeleton. Consequently, induced protrusion of the leading cells formed filopodia and lamellipodia for cell migration (Nagano *et al.*, 2012; Haller, 2012). RAC1 is responsible for the process of malignancy transformation, angiogenesis, invasion, and metastasis (Bid *et al.*, 2013). In addition, Liu and Zhao *et al.* (2016) has been identified that RAC1 increased its expression and suggested that has a role in the pathogenesis of endometriosis.

The increasing of the expression in genes can be caused by genetics or epigenetics modification. Epigenetics is combination between genetic and environmental factors that contribute to the disease etiology (Acosta & Fallin 2016). One of epigenetics modifications that highly contributed to the pathogenesis of endometriosis was DNA methylation, regulates either silencing or activation of gene expressions through hypo or hypermethylation (Koukora *et al.*, 2014; Schübeler, 2015; Gupta *et al.*, 2015).

RAC1 expression has been linked to in diverse phenomenon including cellular transformations, migrations and may relate to pathogenesis of endometriosis. Unfortunately, the evidences of epigenetics modifications such as promoter methylation and its relations to affect mRNA expression of RAC1 in peritoneal and endometrial endometriosis are still limited. Therefore, this study aimed to analyze relation of DNA methylation of RAC1 promoter to mRNA expression level in endometrial and peritoneal endometriosis. Thus, it may explain contributions of methylation level of RAC1 gene as focal adhesion gene in endometriosis pathomechanisms.

MATERIALS AND METHODS

Sample preparation

This cross-sectional study using 20 patients with endometriosis and 20 women as a normal group who underwent in vitro fertilization program with the range of the age in each group was 20-40 years old. The samples were 20 endometrial tissues and 9 peritoneal tissues were taken from the same patient and 20 normal endometrial tissues. Sample collection was conducted according to the protocol of the World Endometriosis Research Foundation (Fassbender *et al.*, 2014).

Samples were collected in the Department of Obstetrics and Gynecology, Faculty of Medicine, University of Indonesia-National Center General Hospital Dr. Cipto Mangunkusumo Jakarta and Endometriosis Centre, Fatmawati Hospital Jakarta. Ethical approval was obtained from

Ethics Committee Faculty of Medicine Universitas Indonesia with protocol number 19-01-0012. This research was conducted at Molecular Biology Laboratory, Integrated Laboratory, and Human Reproduction Cluster, IMERI from October 2018 to February 2019.

Total RNA extraction

Total RNA extraction was performed using Quick-RNA™ MiniPrep (Zymo, USA) according to the manufacture procedures.

cDNA synthesis and mRNA analysis

The cDNA synthesis was performed from total RNA using ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo, Japan). The cDNA samples were then amplified by q-RT PCR using THUNDERBIRD™ SYBR® qPCR Mix (Toyobo, Japan). The total volume for each sample was 6 µL.

cDNA samples were amplified by q-RT PCR using THUNDERBIRD™ SYBR® qPCR Mix (Toyobo, Japan). RAC1 Primers for RT-PCR were designed by Primer Quest Tool and the primers were ordered from Integrated DNA Technologies (Integrated DNA Technologies, USA) Inc. *Forward primer* was GCTAAGGAGATTGGTG CTGTAA and *Reverse* was TCGGATCGCTTCG TCAAAC. Relative quantification of RAC1 mRNA expression using Livak's method was performed (Dorak 2006).

Total DNA extraction

The DNA from tissue samples was extracted using gSYNC™ DNA Extraction kit (Geneaid, Taiwan) according to the protocol.

Promoter methylation analysis

This promoter methylation analysis was following to a previous study that conducted by Darmawi *et al.* (2018). The extracted DNA samples were converted by sodium bisulfite using EpiTect Bisulfite Kit (Qiagen, Germany). The total volume was 140 µL. The bisulfite-converted DNA was amplified by the methylation-specific polymerase chain reaction (MSP). Amplification of methylated and unmethylated primers were performed using MSP method. Primers were designed by MethPrimer program (<http://www.urogene.org/cgi-bin/methprimer/>

methprimer) and the primers were ordered from (Integrated DNA Technologies, USA) Inc. MSP was also used to the positive control samples (EpiTect Methylated Control DNA (human), Qiagen, Germany).

Methylated primers were *Forward-M*: AAGGGATTTGTAGGTTCCGTTAC; *Reverse-M*: CCGAAATTTCTCTAAACTTCGAA and unmethylated primer were *Forward-U*: AAGGGA TTTGTAGGTTTGGTTATG; *Reverse-U*: CTC CCAAAATTTCTCTAAACTTCAA. The size of methylation amplicon was 129 bp, whereas an unmethylation was 132 bp. MSP product was visualized using 3% gel electrophoresis and the band intensity was measured using ImageJ program. The level of promoter methylation of RAC1 gene was determined by comparing band intensity of methylated product to total band intensity from methylated and unmethylated band product.

Statistical analysis

mRNA expression level was analyzed using independent T-test and Mann-Whitney while promoter methylation level using Mann-Whitney test in endometrial, peritoneal endometriosis, and normal endometrium. Correlation between promoter methylation level and mRNA expression level in endometrial endometriosis using Spearman test

RESULTS AND DISCUSSION

The mRNA expression level of RAC1 gene

In this study, we determined the level of expression in RAC1 gene and compared the expression between normal endometrium, endometrial and peritoneal endometriosis. The results showed that RAC1 mRNA expression in peritoneal endometriosis significantly increased to 2.6 fold relative to normal endometrial ($p=0.008$) (Figure 1). While, the mRNA expression in peritoneal endometriosis was significant statistically compared to endometrial endometriosis ($P=0.036$) (Figure 1). Furthermore, the RAC1 expression in endometrial endometriosis found moderate increased 1.3 fold relative to normal endometrial, but it was not significant statistically ($p=0.303$) (Figure 1).

Endometrial of women with endometriosis featured to be abnormal in cellular and molecular mechanism compared to unaffected women (Carvalho *et al.*, 2011). Aberrant of endometrial tissues remodeling, adhesion or attachment, proliferation, and interaction with extracellular matrix and several MMPs are linked to the development of the diseases (Liu and Lang, 2011). It has been known that endometriosis is typically a disease-like cancer that has a metastatic process (Jiang *et al.*, 2012). The metastatic process requires crosstalk between cell and extracellular matrix that linked to dynamic change of actin cytoskeleton. Thus, leading to focal adhesion formation (Jacquemet, 2015). RAC1, is one of focal adhesion genes, has been reported that increased its expression in endometriosis (Liu and Zhao, 2016). The results were also found in our study in the mRNA expression level of RAC1 gene. These results were similar to previous study that expression of RAC1 gene increased in endometrial endometriosis tissues (Ping *et al.*, 2016). Zhao *et al.* (2009) also showed that focal adhesion genes underwent the upregulation of expression in peritoneal endometriosis tissue.

Figure 1. mRNA expression level of RAC1 gene in peritoneal and endometrial endometriosis as well as in normal endometrium.

Promoter methylation level of RAC1 gene

For DNA methylation profiling, we visualized products of MSP in gel electrophoresis from normal endometrium, endometrial and peritoneal endometriosis. The groups were divided into 3 categories of the band such as methylated, unmethylated, and partially methylated if the band appeared on both methylated and unmethylated. We also used K⁺ containing only methylated primers and NTC (non-template control)

containing only unmethylated primers. The K⁺ and NTC were used to ensure the primers are not contaminated.

We collected endometriosis from two distinct tissues, which were 20 endometrial and 9 peritoneal tissues. From endometrial endometriosis, the results showed that 4 unmethylated, 1 methylated, and 15 partials methylated (Figure 2A), while 8 partial methylated and one unmethylated were shown from peritoneal tissues (Figure 2C). On the other hand, in control group that consisted of 20 samples of normal endometrium showed that 5 unmethylated, 1 methylated and 14 partials methylated (Figure 2B).

In this study we also quantified the percentage of promoter methylation of RAC1 gene in normal endometrium, endometrial and peritoneal endometriosis was 25.56%, 29.70%, and 37.86%, respectively. Promoter methylation level of RAC1 gene in peritoneal endometriosis compared to normal endometrium shown significant statistically ($p=0.044$) (Figure 3); but its methylation level was no significantly difference observed in peritoneal compared to endometrial endometriosis ($p=0.119$) (Figure 3). We also showed no significant changes between endometrial endometriosis compared to normal endometrium ($p=0.577$) (Figure 3). A previous study conducted by Meyer *et al.* (2014) reported that the increasing of promoter methylation level in PR gene with methylated allele found in endometriosis lesions, but not in allele of normal endometrium. Other studies by Xue *et al.* (2007) resulted that hypomethylated in promotor region of SF-1 gene and ER- β in lesions affect to the increase of estrogen and progesterone resistance.

Correlation between promoter methylation level and mRNA expression level of RAC1 gene in endometrial endometriosis

Regulation of gene expression requires various components including specific transcription factors that act as regulatory proteins to bind and interact with certain promoter regions (Xin *et al.*, 2008). One of epigenetic modifications that is closely related and most often occurs in the process of gene expression is DNA methylation. According to this result, analysis correlation in endometrial endometriosis shown association between promoter hypermethylation level to

mRNA expression level ($R = 0.014$; $p = 0.952$) (Figure 4).

Unlike hypermethylation that usually promote gene silencing, in contrast this study demonstrated that promoter hypermethylation may enhance mRNA expression. Simultaneously, this result was also in parallel with Weng *et al.* (2015) shown that the increase of EGFR gene expression in gastric cancer was affected by methylation process in CpG promoter regions

and hence leads to the increase of mRNA expression. However, possible explanation for these conditions is that methylation at the promoter close to the transcription start site (tss) triggers conformational changes from chromatin in this area so as to increase gene transcription (Weng *et al.*, 2015). In addition, our suggested that other mechanisms such as histone modification and single nucleotide polymorphisms (SNP) in promoter region play a role in gene expression.

Figure 2. Electrophoresis of MSP product for evaluation of promoter methylation level of RAC1 gene in different tissues. (A) in endometrial endometriosis (EU1-EU4); (B) in normal endometrium (K1-K3); (C) in peritoneal endometriosis (P1-P3). M= methylated, U= unmethylated, K+= positive control, NTC= non template control.

Figure 3. Promoter Methylation level of RAC1 gene in peritoneal endometriosis, endometrial endometriosis, and normal endometrium.

In endometriosis with the presence of endometrial-like tissues outside the uterine cavity is also termed as estrogen-dependent diseases

(Siva *et al.*, 2014). Estrogen is required to promote tissues remodeling and endometrial cell migration by increasing focal adhesion formation and cell membrane morphology alteration (Flamini *et al.*, 2011). This condition triggered protrusion of membrane such as lamellipodia and filopodia which facilitate cell migration (Flamini *et al.*, 2011). Cell migration process is exemplified as in the study of Huang *et al.* (2016) proposes a hypothetical model of breast cancer that activated RAC1 promote cell migration and invasion (Huang *et al.*, 2016). RAC1 gene has the activity to regulate and coordinate in assembly and disassembly process of the actin cytoskeleton (Faix & Weber 2013).

RAC1 gene encodes a protein that is essential for normal cellular activities including phagocytosis, mesenchymal migration, axonal

growth, and differentiation of several types cell (Bid *et al.*, 2013). RAC1 appears to be deregulated in both expression and activity in a variety of tumor cells (Rathinam *et al.*, 2011). RAC1 hyperactivation and overexpression seem closely related to aggressive growth and its activity is important in the processes that underlie malignant characteristic in several different tumor types (Rathinam *et al.*, 2011).

Figure 4. Correlation between promoter methylation level of RAC1 gene and mRNA expression level in endometrial endometriosis.

Overexpression of RAC1 gene led to the pathological condition and it can be found in endometriosis-associated epigenetic mechanisms. The epigenetic process plays a role in regulating gene expression may alter malignancy-associated characteristics. Alteration of methylation on DNA promoter is one of mechanisms related to changes in gene expression observed in endometriosis. However, it is difficult to determine whether alteration in methylation is the cause or consequences of this disease, considering that endometriosis is a multifactorial disease which can be caused by interactions between epigenetics and extrinsic factors.

DNA methylation is a reversible and is influenced by environmental factors, i.e. xenobiotic exposure, social behavior, metabolism and nutritional deficiencies that can have an effect later on, during a period of life development or can be brought down trans generatively (Koukora *et al.*, 2014). In fact, Sofo *et al* reported that there was a correlation of dioxins with epigenetic

changes, one of which is DNA methylation that occurs in endometriosis subjects. Exposure to dioxins is related to changes in gene expression associated with transcription regulation, proliferation, steroid sex metabolism, apoptosis, immune response and cell adhesion.

Since endometriosis is a multifactorial disease, reactive oxygen species (ROS) has been linked to epigenetic alteration in DNA methylation and histone modifications. ROS alters the epigenetic systems through eliminating the DNA and histone methylation marks as well as trigger in changing methylation specific site via regulation of DNMT expression and it will affect to the gene expression (Ito *et al.*, 2017). Thus, it may be concluded that DNA methylation is not a single factor influenced the changes of mRNA expression, but other factors may be contributing to affect it. Based on this research, our future direction is to find a diagnostic biomarker to better understand the pathomechanisms of endometriosis through DNA methylation analysis and perform focal adhesion analysis and cell migration assay to evaluate the RAC1 protein that encourages migration cells in endometriosis.

CONCLUSIONS

An increase of RAC1 mRNA expression play a role in endometrial cell migration to peritoneum and promoter hypermethylation alteration of RAC1 gene associated with expression level of its gene.

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