The mRNA expression and DNA methylation level of fibronectin 1 (FN1) gene encoding focal adhesion molecule in endometrial endometriosis

I E Deraya1, A Hestiantoro2,3, R Muharam2,3, M L S Marwali4, Darmawi5 and Asmarinah2,6*

1Master Program in Biomedical Sciences, Faculty of Medicine, Universitas Indonesia, Salemba, Central Jakarta, Jakarta 10430, Indonesia
2Human Reproductive, Infertility and Family Planning Cluster, IMERI, Faculty of Medicine, Universitas Indonesia, Salemba, Central Jakarta, Jakarta 10430, Indonesia
3Department of Obstetrics and Gynecology, Faculty of Medicine, Universitas Indonesia, Salemba, Central Jakarta, Jakarta 10430, Indonesia
4Endometriosis Center, Fatmawati General Hospital, South Jakarta, Jakarta 12430, Indonesia
5Department of Histology, Faculty of Medicine, University of Riau, Pekanbaru 28133, Indonesia
6Department of Medical Biology, Faculty of Medicine, Universitas Indonesia, Salemba, Central Jakarta, Jakarta 10430, Indonesia

*Corresponding author: asmarinah.si@gmail.com

Abstract. Endometriosis is characterized by the presence of endometrial-like tissues located outside the uterine cavity. The expression of focal adhesion genes including FN1 gene increased in endometriosis. Epigenetic factors play a role in the alteration of expression that lead to the pathological conditions. This study aimed to analyze the mRNA expression and promoter methylation level of FN1 gene as an epigenetic mechanism in endometriosis. Forty of the total samples from endometrial patients and normal were used. The DNA and RNA were isolated, DNA was converted using sodium bisulfite procedure, amplified by MSP method. Promoter methylation level was determined by intensity of the bands that arose in gel electrophoresis using ImageJ software. The RNA was reverse transcribed into cDNA and was amplified using RT-qPCR. The mRNA expression level of FN1 gene was higher in endometrial endometriosis compared to normal, but not significant statistically (p=0.63). There was a significant difference methylation level of FN1 gene in endometrial endometriosis compared to normal endometrium (p=0.022). There was positive correlation between promoter methylation level to its mRNA expression in endometrial endometriosis (r=0.080; p=0.736). DNA methylation alteration in promoter FN1 gene was not to be caused by the increasing of mRNA expression.

Keywords: DNA methylation, endometriosis, focal adhesion, FN1

1. Introduction

Endometriosis is a benign gynecological disorder, which is characterized by the presence of...
endometrial-like tissues that grows outside the uterine cavity at exact locations include intraperitoneal and extraperitoneal [1-3]. Endometriosis often occurs in women during their reproductive age with 10% prevalence in general population [3, 4] and following by several main symptoms such as chronic pelvic pain, infertility or subfertility, dysmenorrhea, and dyspareunia [4, 5]. Etiopathogenesis endometriosis could explain through the most acceptable and well-known theory called retrograde menstruation [6]. The theory proposed that there is a backflow of menstrual blood containing endometrial cell debris that moves through the fallopian tubes, enters the ovary and the pelvic cavity then implants and proliferates [6]. Consequently, endometrial cells play a role in the formation of ectopic lesions [7, 8]. Retrograde menstruation occurs in 76-90% of women of reproductive age, but only about 10% which develops into endometriosis [6, 8]. It can be caused by several factors such as genetics, hormonal, immune system, environment [9], and recently some researchers have been proved that epigenetics, particularly DNA methylation contributes to endometriosis [10, 11]. Epigenetics such as DNA methylation could regulate the activation and inactivation of the genes in the transcription process [12].

Endometriosis shares number of similarities with malignant diseases [3]. These include cellular adhesion and invasion, neoangiogenesis, resistance to apoptosis, increasing proliferation, abnormality in cell morphology, DNA aneuploidy, and loss of heterozygosity [3, 5]. Studies on searching in candidate genes that contribute to pathogenesis endometriosis have been done through Genome-Wide Association Studies (GWAS) [5]. There were several genes deregulated in both ectopic and eutopic endometrium, one of which is FN1. FN1 found as susceptible loci and affects the development of endometriosis [13-16]. The previous study conducted by using microarray method showed FN1 that involved in focal adhesion genes increased in endometrial endometriosis [17]. FN1 is a member of the Fibronectin family, which is widely expressed by several cell types and is involved in the process of cell migration, cell proliferation, cell adhesion and participating in extracellular membrane changes. FN1 has a role in embryogenesis, wound healing, blood coagulation, host defense, and metastasis [18].

In recent years, the study, which explored focal adhesion genes contribute to the progression of endometriosis development remains unclear. So that, this study aimed to analyze the mRNA expression level of FN1 gene as a focal adhesion gene as well as the DNA methylation level of its gene that may involve in endometriosis pathogenesis.

2. Material and methods
This study was designed as cross-sectional with the total of samples were 20 endometrial tissues women suffered endometriosis by laparoscopic and 20 normal endometrial tissues from women without endometriasis as control, who underwent in vitro fertilization program. Samples were obtained at the Department of Obstetrics and Gynecology, Faculty of Medicine, University of Indonesia-National Center General Hospital Dr. Cipto Mangunkusumo Jakarta and Fatmawati Hospital Jakarta. Ethical approval was obtained. This research was conducted at Molecular Biology Laboratory, Integrated Laboratory, and Human Reproduction Cluster, Indonesian Medical Research Institute (IMERI), Universitas Indonesia.

The DNA and RNA from endometrial tissues were isolated. Total RNA from tissue samples was extracted using Quick-RNA™ MiniPrep (Zymo Research), then reverse transcribed into cDNA using ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo). cDNA samples were amplified by q-RT PCR using THUNDERBIRD™ SYBR® qPCR Mix (Toyobo). Relative quantification of FN1 mRNA expression using the 2−ΔΔCT value of Livak’s method was performed and statistically analyzed using independent T-test. Correlation between promoter methylation level and mRNA expression level in endometrium endometriosis was analyzed by using Spearman test.

DNA was extracted using Geneid® Genomic Extraction Kit (Biotech Ltd), then converted by sodium bisulfite using Epitect Bisulfite Kit (Qiagen 59140). For DNA methylation profiling, the bisulfite–converted DNA was amplified by the methylation-specific polymerase chain reaction (MSP) method. Methylated and unmethylated primers for FN1 gene amplification in the promoter region were designed by MethPrimer program. The length of methylation and unmethylation amplicon was
184 bp in size. MSP product was visualized in gel electrophoresis and the intensity of the band was measured by ImageJ program. The level of promoter methylation was determined by comparing the band intensity of methylated product to totally band intensity from methylated and unmethylated products. Statistical analysis was performed using Mann-Whitney test.

3. Results
The mRNA expression level of *FN1* gene in endometrial endometriosis compared to normal endometrial tends to increase but it was not significant statistically (p = 0.63) showed in figure 1. Based on gel electrophoresis of MSP products, it was categorized into 3 status of promoter methylation, i.e. methylated, unmethylated, partial methylated if the band appeared in both methylated and unmethylated band (figure 2). The methylation status in promoter *FN1* gene showed that from 20 samples in endometrial endometriosis, 15 samples were partially methylated and 5 samples were unmethylated, whereas from 20 samples in normal endometrial showed that 3 samples were methylated, 1 sample unmethylated, and 16 samples were partially methylated.

![Figure 1](image1)

**Figure 1.** The mRNA expression level of *FN1* gene in endometrial endometriosis compared to normal endometrial.

![Figure 2](image2)

**Figure 2.** Electrophoresis of MSP product of *FN1* gene in endometrial tissue from 2 women with endometriosis and 5 women without endometriosis as control. M= methylated, U= unmethylated, K+= positive control, K-= negative control, EU= endometrial endometriosis, K= control.
The mean of promoter methylation percentage in endometrial endometriosis was 37.95% and in normal endometrial was 59.22%. The methylation level of \(FN1\) gene resulted that in endometrial endometriosis compared to normal endometrium was 22% lower and it was significant statistically \((p=0.022)\) (figure 3). The correlation between promoter methylation level and mRNA expression showed positive correlation with \(p=0.736\) and \(R=0.080\) (figure 4).

**Figure 3.** Promoter methylation percentage of \(FN1\) gene in endometrial endometriosis compared to normal endometrial.

**Figure 4.** Correlation between promoter methylation level of \(FN1\) gene and mRNA expression level in endometrial endometriosis.

4. Discussion

Endometriosis is a multifactorial disease caused by several factors such as the influence of genetics, hormonal, immune system, environment [9] and recently epigenetics, particularly DNA methylation contribute to endometriosis [10, 11]. DNA methylation can occur in CpG shores, gene body, repetitive sequence, and mostly in the promoter region with a high density of CpG islands [19]. Based on our results showed that DNA methylation level in the promoter \(FN1\) gene was lower in endometrial endometriosis compared to normal endometrial (figure 3). It was interpreted that the methylation level of \(FN1\) gene was decreasing and it referred to hypomethylation. The promoter of the genes containing a high density of CpG islands could have a condition called hypermethylation and hypomethylation [10]. Hypermethylation associated with gene silencing resulting in decreasing mRNA expression while hypomethylation induced activation of transcription [19, 20]. On the other hand, this result
showed that in the mRNA expression level of \( FNI \) gene tends to increase compared to normal endometrium but statistically not significant (figure 1). For analysis correlation resulted in positive correlation and it interpreted that the higher the methylation affected in the increasing of the mRNA expression level (figure 4). This result was not usually happened. Generally, hypermethylation caused decreasing the gene expression.

DNA methylation which part of epigenetics control mechanism for the gene expression is typically inheritable and specific in certain tissues. Epigenetics could regulate activation or inactivation of several genes simultaneously [21]. Since endometriosis caused by several factors, epigenetics particularly DNA methylation not always regulate gene expression. Other mechanisms, for example histone modification and genetic variants also participate in this process. Histone modification such as acetylation in lysine residue that is catalyzed by histone acetyltransferase (HAT) affects chromatin remodeling resulting in access for transcription factors, consequently activation in mRNA transcription [22]. Furthermore, genetic variants such as single nucleotide polymorphisms (SNPs) in promoter, introns, exons, and regulatory sequences could affect gene expression [23].

The results of mRNA expression will form proteins through several modifications of mRNA. These proteins have different functions and interact with one another as found in the signaling model of fibronectin in breast cancer [24]. Fibronectin as a ligand will bind to integrins so that it will recruit and activate proteins such as FAK, Src, Paxillin and end up activating of RAC1 proteins. The signaling showed that interaction between Fibronectin and RAC1 would facilitate the process of cell migration and invasion [24], through forming lamellipodia and filopodia. Cell migration involves many proteins that begin with the production of mRNA through the process of regulating gene expression. This process can be regulated by epigenetic modification, one of which is DNA methylation. Changes in the pattern of DNA methylation could have implications for changes in mRNA expression that generated to form proteins and could lead to pathological conditions.

The pathological conditions can be seen in a gynecological disorder such as endometriosis. Endometriosis is a benign estrogen-dependent and is characterized by the presence of endometrial like-tissues in ectopic sites [2, 25]. Endometriosis has a number of similarities with cancer in cellular and molecular features [26]. Cancer has a metastatic process and it also occurs in endometriosis that lesions can be found in endopelvic and extra pelvic throughout the body such as the rare case in the brain, pleura, liver, and diaphragm [27, 28]. Endometriosis showed an abnormality in proliferation, endometrial cell remodeling, invasion and interaction with extracellular matrix (ECM) [4, 26, 29]. As the development of the disease, endometriotic cells need to establish cell-ECM interaction with the lining of implant sites such as peritoneal lining, through adhesion molecules and their ligands [2]. Fibronectin is classified into focal adhesion genes and encoded a component of extracellular matrix [17]. Focal adhesion is the molecular bridge mediating two-way crosstalk between extracellular matrix and cytoskeleton [17]. It promotes many cellular activities include proliferation, adhesion, cell migration and invasion. Fibronectin has been identified into 2 forms i.e. cellular fibronectin and plasma fibronectin [30].

Fibronectin can act in both physiological and pathological conditions. In physiological conditions, these proteins function in embryonic development, migration, proliferation, cell growth, blood coagulation, host defense, and tissue remodeling [31]. During the process of tissue remodeling such as tissue repair due to injury, the expression of fibronectin depends on the type of isoform [32]. This process requires assembly and arrangement of fibronectin into fibrils, which are the initial components of the extracellular matrix [33]. Conversely, in the pathological process, fibronectin can regulate malignant transformation including cell growth and development, invasive migration, and metastasis [34] as well as cell adhesion and differentiation [33]. FN1 is known to be widely expressed in various cancer, thus acting as an essential factor for cancer development. FN1 has shown involvement in cell proliferation, migration, and participation in changes in extracellular matrix through signaling with integrins in several types of cancers including squamous oral cancer, nasopharyngeal cancer, ovarian cancer, kidney cancer, and thyroid cancer [18].
5. Conclusion
There was a significant decrease in DNA methylation level of FN1 gene but it did not correlate with the increasing in its mRNA expression in endometrial endometriosis tissues.

Acknowledgments
We are thankful to HIBAH PITTA 2018 and 2019 for funding this research.

References
[1] Borghesea B, Zondervanc K T, Abrao M S, Chaprona C and Vaiman D 2017 Clin. Genet. 91 254–64
[2] Adachi M, Nasu K, Tsuno A, Yuge A, Kawano Y and Narahara H 2011 Eur. J. Obstet. Gynecol Reprod. Biol. 155 85–8
[3] Bürkle B, Notscheid N K, Scheich J, Hefler L, Tempfer C B, Horvat R and Reznicek G A 2013 Eur. J. Obstet. Gynecol. Reprod. Biol. 169 370–5
[4] Aznaurova Y B, Zhumataev M B, Roberts T K, Aliper A M and Zhavoronkov A A 2014 Reprod Biol. Endocrinol. 12(50) 1–25
[5] Fung J N, Rogers P A W and Montgomery G W 2015 Biol. Reprod. 92(4) 1–12
[6] Sourial S, Tempest N and Hapangama D K 2014 Int. J. Reprod. Med. 1–10
[7] May K E, Villar J, Kirtley S, Kennedy S H and Becker C M 2011 Hum. Reprod. 17(5) 637–53
[8] Soo H A, Vinay S and ChandrakanT 2017 Fertil. Steril. 107(3) 523–32
[9] Gupta S, Harlev A and Agarwal A 2015 Endometriosis A comprehensive update. (New York: Springer) p 7–15
[10] Nasu K, Kawano Y, Tsukamoto Y, Takano M, Takai N, Li H, Furukawa Y, Abe W, Moriyama M and Narahara H 2011 J. Obstet. Gynaecol. Res. 37(7) 683–95
[11] Koukoura O, Sifakis S and Spanidios D A 2016 Mol.Med.Rep. 13 2939–48
[12] Chan R W, Ng E H and Yeung W S 2011 Am. J. Pathol. 178(6) 2832–44
[13] Pagliardini L, Gentilini D, Panina-Bordignon P, Busacca M, Candiani M and Di Blasio A M 2013 J. Med.Genet. 50 43–6
[14] Painter J N, Anderson C A, Nyholt D R, Macgregor S, Lin J, Lee S H, Lambert A, Zhao Z Z, Roseman F, Guo Q and Gordon S D 2011 Nat. Genet. 43 51–4.
[15] Nyholt D R, Low S K, Anderson C A, Painter J N, Uno S, Morris A P, MacGregor S, Gordon S D, Henders A K, Martin N G and Attia J 2012 Nat. Genet. 44:1355–9
[16] Rahmioglu N, Nyholt D R, Morris A P, Missmer S A, Montgomery G W and Zondervan K T 2014 Hum. Reprod. Update. 20 702–16
[17] Ping S, Ma C, Liu P, Yang L, Yang X, Wu Q, Zhao X and Gong B 2016 Arch. Gynecol. Obstet. 293 797–804
[18] Cai X, Liu C, Zhang T N, Zhu Y W, Dong X and Xue P 2017 J. Cell. Biochem. 119(6) 4717-28
[19] Portela A and Esteller M 2010 Nat. Biotechnol. 28(10) 1057–68
[20] Gronbæk K, Hother C and Jones P A 2007 Apmis. 115(10) 1039–59
[21] Chan R W, Ng E H and Yeung W S 2011 Am. J. Pathol. 178(6) 2832-44
[22] Xiaomeng X, Ming Z, Jiezhi M and Xiaolin GF 2013 Arch. Gynecol. Obstet. 287 487–94
[23] Samartzis E P, Noske A, Samartzis N, Fink D and Imesch P 2013 Reprod. Sci. 20(12) 1416–22
[24] Huang Y S, Chang C C, Lee S S, Jou Y S and Shih H M 2016 Oncotargeter. 7(28) 1–11
[25] Zondervan K T, Rahmioglu N, Morris A P, Nyholt D R, Montgomery G W, Becker C M and Missmer S A 2016 In Semin. Reprod. Med. 34 242–54
[26] Klemmt P A, Carver J G, Koninkecx P, McVeigh E J and Mardon H J 2007 Hum. Reprod. 22(3)139–47
[27] Burney R O and Giudice LC 2012 Fertil. Steril. 98(3) 511–19
[28] Machairiotis N, Stylianaki A, Dryllis G, Zarogoulidis P, Kouroutou P, Tsiamis N, Katsikogiannis N, Sarika E, Courcoutsakis N, Tsiouda T and Gschwendtner A 2013 Diagn. Pathol. 81(94) 1–12
[29] Qiao-Ying J and Rui-Jin W 2012 *Gynecol. Endocrinol.* **28**(7) 562–67
[30] Bingham R J and Potts J R 2010 *Structure.* **18**(6) 660–61
[31] Yousif N G 2014 *Cell. Biol. Int.* **38** 85–9
[32] To W S and Midwood K S 2011 *Fibrogenesis Tissue Repair.* **4**(21) 1–17
[33] Xu J and Mosher D 2011 *Fibronectin and Other Adhesive Glycoproteins. In: Mecham R, editors. The Extracellular Matrix: an Overview* (Berlin:Springer) p 41–79
[34] Cao Y, Liu X, Lu W, Chen Y, Wu X, Li M, Wang X A, Zhang F, Jiang L, Zhang Y and Hu Y 2015 *Cancer Letters.* **360**(2) 141–50