

DNA hypomethylation of the nuclear receptor subfamily 5 (NR5A1) gene promoter is associated with endometriosis among women in north west of Iran

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Abstract

Background and objectives: Endometrial tissue growth and its activity outside the uterus cause endometriosis. It has been suggested that various epigenetic deviations play a major role in the pathogenesis of endometriosis. *Steroidogenic factor 1 (SF-1; NR5A1)* is an essential transcription factor for estrogen biosynthesis in endometrial cells. The expression of *SF-1* in endometriosis and lack of expression in normal endometrium is primarily determined by its promoter methylation. Here, we aimed to compare the methylation status of the *SF-1* gene promoter region in women with endometriosis in comparison to healthy subjects.

Methods: In the present case-control study, DNA was extracted from 25 endometrial tissue samples from women with endometriosis and 5 normal post-hysterectomy endometrium tissues which were collected from Tabriz hospitals including Vali-e-Asr, Taleghani, 29 Bahman and Shams in 2016. The obtained DNA samples were subjected to Bisulfite-treatment. Finally, the status of *SF-1* gene promoter methylation was evaluated by methylation specific PCR method. Statistical analyses including descriptive and inferential statistics were conducted using tables, bar charts by statistical software SPSS version 20 and independence test.

Results: The methylation status of *SF-1* gene promoter was decreased significantly in endometriosis samples ($P < 0.05$).

Conclusion: *SF-1* gene promoter hypomethylation could increase the relative expression of *SF-1* gene in endometriosis which may lead to the development or progression of the disease.

Keywords: Endometriosis, Promoter Methylation, Steroidogenic Factor 1 Gene, NR5A1

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Introduction

Endometriosis is an estrogen-dependent inflammatory disease which affects approximately 5 to 10 percent of women in the United States during reproductive ages. It is characterized by the presence of endometrial tissue outside the uterine cavity, primarily affecting the peritoneum and the ovary. Its main clinical features are chronic pelvic pain, pain during intercourse and infertility (1). Endometriosis could be resulted due to various biochemical or anatomical abnormalities in the function of the uterus. In most cases, menstrual bleeding in women with endometriosis is associated with the development of the disease (2). In contrast, there could be mechanisms for the development of endometriosis other than anatomical disorders, such as exposure to peripheral toxins or strong estrogen during fetal development (3). Clinical evidence suggests a major role for estrogen and progesterone in the development of endometriosis. Excessive estrogen production and progesterone resistance, therapeutic targeting of aromatase in estrogen biosynthesis pathway and cyclooxygenase-2 (COX-2) in prostaglandin pathway are effective in pelvic pain in endometriosis (4-7). In addition to these three important objectives, epigenetic markers could result in SF-1 and estrogen receptor beta (ER- β) overexpression through nuclear receptors (8, 9). The origin of most diseases is multifactorial, which is a pool of environmental factors and epigenetic changes. Evidence shows that endometriosis is also largely influenced by exogenous factors due to epigenetic alterations (10).

Epigenetic changes could be classified into three main categories: covalent histone changes, non-coding RNAs and DNA

methylation. Methylation is one of the most important epigenetic changes which include the addition of methyl groups to cytosine units in the regions of CG dinucleotide of the DNA (also called CpG) (11). The effective enzymes in methylation are DNA methyl transferase (DNMTs) which use S-adenosylmethionine as a methyl donor and add the methyl group to the cytosine ring (12). Generally, when cytosine is expanded within CpG islands, the gene promoter could be methylated and as a result the gene would be turned off by methylation which is called "hypomethylation" (13). During the process of inhibiting promoter by methylation, transcription factors and RNA polymerase preferably bind to a protein called methyl cytosine binding protein (MeCP) and inhibit transcription (14). In endometriosis, the transcription stimulatory factor (SF-1) and transcription inhibitor (COUP-TF) compete for the same binding site in the aromatase promoter region. COUP-TF is expressed in both normal endometrium and endometriosis, while SF-1 is exclusively expressed in endometriosis (10).

Several studies also point to the direct role of SF-1 in gender determination. The protein encoded by this gene is a transcriptional activator which is involved in sex determination. A defect in this gene results in a XY sexual reversal with or without adrenal failure as well as adrenal insufficiency without defective ovary (15). Recent studies showed that SF-1 in addition to Sry acts on a specific gonadal enhancer to increase the expression of Sox-9 and also to activate the transcription of Sry coding gene which plays an important role in the genetic network that guides gender determination and differentiation (16, 17).

NR5A1 (nuclear receptor subfamily 5 group A member1; ID: 2516) as a transcription factor is also known with other names such as *SF-1* (steroidogenic factor 1) and *Ad4BP*. It is a member of the nuclear receptor family which is encoded by the *NR5A1* gene in huma (18, 19). *SF-1* is a key factor for steroid biosynthesis and is responsible for inducing the expression of *STAR* and *Cyp19A1* which is an aromatase coding agent (10). Aromatase facilitates the last stage of estrogen production through steroid C19 conversion. It has been reported that *SF-1* mRNA and protein levels are significantly overexpressed in endometriosis in comparison to the normal endometrium which is associated with the severity of endometriosis (20). The aim of the present study was to introduce the ability to detect DNA methylation as a strong diagnostic test with a high potential for clinical use. Due to the obstacles in the diagnosis of endometriosis, *SF-1* gene hypomethylation could be used as a biomarker for accurate diagnosis of this disease. Moreover, DNA methylation pattern could be used to classify endometriosis, select a therapeutic approach, decide on the appropriate dosage, and predict the response.

Materials and Methods

It was a case-control study conducted on the samples from patients with confirmed endometriosis according to the microscopic examinations of the pathologic tissues. None of the patients were under medical treatment. A total of 25 age-matched endometriosis tissue samples and 5 normal endometrial tissues from individuals with uterine prolapse under hysterectomy operation were enrolled from Vali-e-Asr (international), Taleqani and 29 Bahman hospitals in Tehran and Shams hospital in Tabriz since August to February of

2016. The pathologic endometriosis samples were obtained from laparoscopic surgery and confirmed by gynecologist during or before surgery with ultrasound evidence and MRI. The samples were kept in RNAlater and transferred to the molecular genetic laboratory of the infertility treatment center, the academic center for education, culture and research of Tabriz, East Azerbaijan, Iran and stored at -20 °C until DNA extraction and the subsequent experiments. DNA extraction was performed by salting out method (21). The concentration and quality of extracted DNA were determined using BioPhotometer plus spectrophotometer (Eppendorf, Germany).

Methylation specific PCR method

A specific PCR method was used to study the DNA methylation pattern in *SF-1* gene promoter. Methylation points on the genomic DNA are deleted during replication and not replicated by DNA polymerase. Thus, the DNA template should be chemically modified using sodium bisulfite to preserve the methylation data before replication. Sodium bisulfite changes non-methylated cytosine to uracil, while 5-methyl cytosine is resistant to these changes. Following the amplification of the bisulfited pattern, different products of methylated and non-methylated patterns are obtained. GC-rich fragments with methylated patterns and preserved methylated cytosine and also the fragments with low GC-content are recognized as non-methylated, in which all cytosines have changed to uracil. All samples were bisulfited using EZ DNA Methylation-Gold™ (Zymo Research, CA, USA) according to the manufacturer's protocols. Specific primers were used to study methylation of *SF-1* promoter regions (Table 1). Two pairs of primers were required for each gene; one pair to check methylation and the other pair to check the lack of

methylation. Non-methylated DNA which is often found in all peripheral blood mononuclear cells (while their promoter is not methylated) was used as negative control (22). First, the lymphocytes should be isolated from the patient's blood. The most convenient way is to use solutions which provide a high concentration density. One of the most widely used solutions is Ficoll which was used in the present study to isolate PBMCs. Methylated DNA control (100%) using EpiTect Control DNA methylated kit (QIAGEN, USA) was used as positive control. The reaction for each sample requires two separate tubes for MSPCR to evaluate the methylation or non-methylation of the genes with different specific primers in each tube.

Briefly, each 12 μ L PCR reaction mixture for a *Meth* primer contained 6 μ L of 1X PCR Master Mix (Ampliqon, Denmark), 3.5 μ L dH₂O, 100 ng of DNA template, 0.25 μ L of each primer pair (10 pM). Regarding the *Unmeth* primer, each reaction mixture contained 6 μ L of 1X PCR Master Mix (Ampliqon, Denmark), 3 μ L dH₂O, 100 ng of DNA template and 0.5 μ L of each primer pair (10 pM). PCR was performed under the following temperature conditions; an initial denaturation of 95 °C for 10 minutes followed by 45 cycles including 95 °C for 20 seconds, the annealing temperature of 65 °C for 15 seconds in *Meth* primer and 63 °C for 15 seconds in *Unmeth* primer reaction mixtures followed by 72 °C for 20 seconds. The PCR product was electrophoresed in 2% agarose gel and visualized directly under the UVITEC gel doc (Cambridge, England). All samples were visualized as single bands after electrophoresis which demonstrates the quality and accuracy of the PCR function. The 99 bp *Meth* primer PCR amplification product and the 104 bp *Unmeth* were detected (Figure 1). SPSS version 20 was used to

analyze data statistically. Independence test was used to study the research hypothesis.

Results

PCR product with the *Meth* primer (99 pairs) was observed in every 5 healthy subjects, which showed that all of them were hyper-methylated (Figure 1). The PCR product of *Meth* primer was reported to be positive among 7 individuals out of 25 endometriosis patients, which represented them as hyper-methylated (Figure 2). The *Meth* control (100% of methylated) is also shown in figure 2.

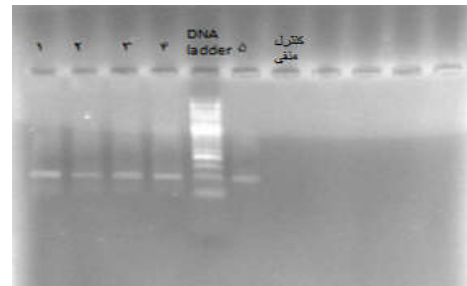


Figure 1. PCR product of *Meth* primer in healthy subjects

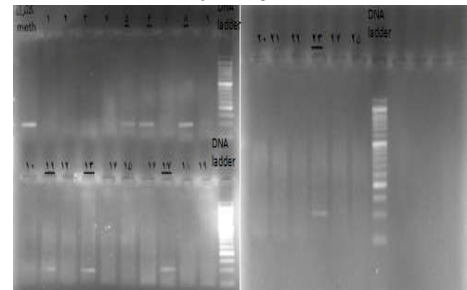


Figure 2. PCR product of *Meth* primer in patients

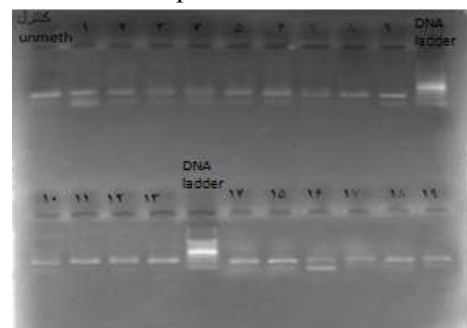


Figure 3. PCR product of *Unmeth* primer in patients

The PCR product of *Unmeth* primers (104 bp) could be visualized in all 25 patients, which suggests that all patients are hypo-methylated (Figure 3 and 4A). The *Unmeth* control (zero percent of methylation) is also observed in figure 3. The PCR product of healthy subjects with *Unmeth* primer indicated that one of the healthy subjects was hypo-methylated (Figure 4B).

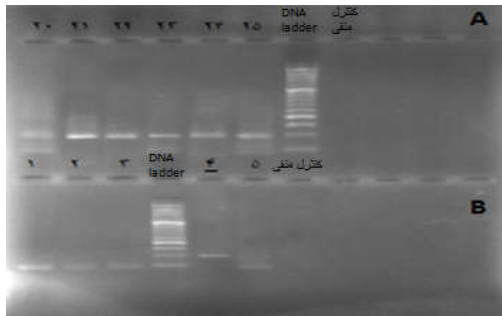


Figure 4. The PCR product of the patient with *Unmeth* primer (A) and healthy subjects with *Unmeth* primer (B)

Among patients, seven out of 25 samples were positive with both *Meth* and *Unmeth* primers which showed that these samples were partially methylated (50% of methylation which were positive with both primers). Among healthy subjects, one out of five samples was positive with both *Meth* and *Unmeth* primers which represented partial methylation in the mentioned sample. Table 2 demonstrates the distribution frequency of endometriosis and normal endometrium tissue samples regarding the "methylation of the *SF-1* gene". Moreover, the results of the independence test to analyze the above assumptions are presented in Table 3. While the *P*-value is lower than 0.05, the methylation status of *SF-1* gene promoter is significantly different between endometriosis samples and normal endometrial tissues.

Table 2. Distribution of *SF-1* methylation in patients and healthy subjects

	Hyper-methylated		Partially methylated		Hypo-methylated	
	N	%	N	%	N	%
Healthy	5	100	1	20	1	20
Patient	7	28	7	28	25	100

Table 3. the results of the independence test

Test	Statistics	Degree of freedom	<i>P</i> -value
Chi-square	9.158	2	0.010
Likelihood ratio	8.428	2	0.015

Discussion

Estradiol biosynthesis relies on facilitating the entry of cholesterol into the mitochondria followed by six enzyme stages of which the aromatase is the key enzyme and at the final stage C19 steroid is converted to estrogen. Since the presence of *SF-1* gene in endometrial stromal cells and its absence in endometrial stromal cells is a key event for aromatase expression, better understanding of its molecular mechanism and *SF-1* gene expression regulation may provide new opportunities for targeted treatment of endometriosis. It has previously been reported that the methylation status in the promoter and the exon I region of some genes may determine the transcription activity, mRNA levels and/or protein (28-32). Hyper-methylation of CpG islands may inhibit transcription through interfering with employing or function of basal transcription factors or co-activators. Moreover, hyper-methylation of CpG di-nucleotides in the vicinity of the transcriptional regulatory region may also suppress these genes by employing methylation dependent DNA-binding proteins (28-30). Understanding the function of these proteins (especially MeCP2)

suggests that suppressing transcription by methylation is due to the binding of these proteins to CpG which prevents the binding and function of transcription factors (31, 32). It is also suggested that different methylation status of CpG islands in the promoter and exon I of the *SF-1* gene may be a key mechanism for the expression of *SF-1* mRNA in endometrial stromal cells and also may contribute to STAR, aromatase and other important genes for estrogen biosynthesis in endometriosis (9).

Several studies have shown that DNA methylation in the promoter region is associated with transcription (33-37). A recent study has linked the methylation and mRNA expression of *SF-1* in endometriosis and endometrial cells and expressed that normal endometrial cells do not express *SF-1* and proximal promoter was found to be hyper-methylated. However, the endometriosis cells in which the SF-1 is expressed, similar regions are hypo-methylated (9). In addition, many of the relevant sites for binding transcription factors are probably located outside the gene promoter especially in intron (38-41).

The promoter sequence was found in our study and our results in this area fully match the results obtained in previous studies with similar cases. In the present study, all 25 patients were hypo-methylated and the healthy group was hyper-methylated. Partial methylation was observed among 7 patients and 1 healthy subject which suggests that hypo-methylation results in the expression of *SF-1* gene, which also causes the expression of the aromatase encoding gene (CYP19A1), and steroid genes (including STAR, SCC, HSD3B2 and 17-hydroxysteroid-17-lyase) and ultimately the production of estrogen leads to endometriosis.

Hoivik et al. also reported that this region is hypo-methylated in SF-1 expressing human cells and hyper-methylated in non-expressing cells (42). In consistent with Hoivik et al., the endometriosis tissue was hypo-methylated and the normal endometrial tissue was hyper-methylated in the present research study. They also found that the state of DNA methylation and enhancers is highly related to its activity. Considering the importance of the SF-1 gene in the onset of disease, its promoter region is also hypo-methylated in our study and hyper-methylated in healthy subjects. It suggests that in case of hyper-methylation, *SF-1* gene is not expressed; the CYP19A1 gene (aromatase encoder) and the cascade of steroidogenic genes are silenced and not expressed.

There is a positive correlation between increasing the methylation of CpG islands in the exon II / intron III region and the expression of *SF-1* in endometriosis, because this sequence is not significantly expressed in normal endometrial stromal cells and remains hypo-methylated. Interestingly, the hyper-methylation of this exon/intron region activates *SF-1* mRNA in endometriosis cells, which in turn the gene expression is turned off. The CpG islands in the exon II/intron III region may possess an extinguishing regulatory element. Subsequently, the methylation of this element neutralizes its silencing function and increases the expression of *SF-1* (43).

Xue et al. reported that *SF-1* mRNA levels were significantly higher in endometriosis stromal cells in comparison to endometrial stromal cells. The bisulfite sequence around the CpG islands showed a strong association with the overexpressed *SF-1* mRNA expression in endometrial cells. Therefore, these CpG islands play an important role in regulating the expression of SF-1 (44).

Investigating the genomic profile of endometriosis patients compared with the normal endometrium, Yamagata et al. revealed that *NR5A1* and *STAR* genes were hypo-methylated while *STAR6* and *HSD17 β 2* genes were hyper-methylated. They found different methylation profiles in different genes of the disease. Generally, DNA methylation in endometriosis has a significant effect on gene expression and progression (45).

Our findings also revealed that the *SF-1* gene (*NR5A1*) was hypo-methylated in the promoter region of endometriosis cells. It may affect the steroidogenic genes cascade, including the *STAR* genes and regulate the conversion of cholesterol to androstenedione by hypo-methylation. On the other hand, *SF-1* (*NR5A1*) hypo-methylation leads to its overexpression, its effect on aromatase and conversion of androstenedione to estrone. *HSD17 β 2* gene regulates estrogen to estradiol conversion and endometriosis development. *STAR6* is also a surface receptor for retinol absorption. The selected genes are highly pathogenic genes and are significantly related to each other. In the study conducted by Yamagata et al., the genomic signature and their transcriptome was investigated in addition to the methylation status which reflected a pattern of general expression that introduced methylation to be more successful in predicting the pathogenesis of endometriosis.

Conclusion

The difference in the methylation status of *SF-1* gene the promoter in the endometriosis tissue sample was statistically significant in comparison to the normal endometrial tissue. We believe that this kind of novel information would be useful for future research on

therapeutic and preventive approaches for endometriosis.

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هایپومتیلاسیون DNA در پروموتور ژن *nuclear receptor subfamily 5 group A member1 (NR5A1)* مرتبط با اندومتريوز زنان در شمال غرب ایران

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چکیده:

زمینه و هدف: رشد بافت آندومتر و فعالیت آن در خارج از رحم باعث ایجاد اندومتريوز می شود. شواهد نشان می دهد که انحرافات مختلف اپی ژنتیکی نقش اساسی در پاتوژنز اندومتريوز بازی می کند. *SF-1* یک عامل رونویسی ضروری، برای بیوسنتز استروژن در سلول های اندومتريوتیک است. بیان *SF-1* در اندومتريوز و عدم بیان آن در آندومتر در درجه اول توسط متیلاسیون پروموتور آن تعیین می شود. مطالعه حاضر با هدف مقایسه وضعیت متیلاسیون ناحیه پروموتوری ژن *SF-1* در زنان مبتلا به اندومتريوز و سالم انجام گرفت.

روش بررسی: در مطالعه موردی - شاهدهی حاضر از ۲۵ نمونه بافت اندومتريوتیک از زنان مبتلا به اندومتريوز و ۵ نمونه بافت آندومتر طبیعی از افراد تحت عمل هیستریکتومی که از بیمارستان های ولیعصر، طالقانی، ۲۹ بهمن و شمس شهر تبریز در سال ۱۳۹۵ جمع آوری گردید، DNA استخراج شد. نمونه های DNA حاصل بی سولفیت شده و در نهایت وضعیت متیلاسیون پروموتور ژن *SF-1* توسط *methylation specific PCR* مورد ارزیابی قرار گرفت. آنالیز آماری شامل دو قسمت آمار توصیفی و آمار استنباطی با استفاده از نرم افزار آماری spss نسخه ۲۰ و آزمون استقلال انجام شد.

یافته ها: میزان متیلاسیون پروموتور ژن *SF-1* در نمونه های اندومتريوتیک سطح کاهش یافته و با اندومتريوز ارتباط معنی داری دارد ($P < 0.05$).

بحث و نتیجه گیری: هیپو متیلاسیون پروموتور ژن *SF-1* می تواند باعث افزایش نسبی بیان ژن *SF-1* در بافت اندومتريوز شود که این افزایش بیان در نهایت ممکن است منجر به ایجاد یا پیشرفت بیماری شود.

کلمات کلیدی: اندومتريوز، متیلاسیون پروموتور، ژن NR5A1، Steroidogenic factor 1

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