

Epigenetic aberration of gene expression in endometriosis

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1. ABSTRACT

Endometriosis is an estrogen-dependent inflammatory disease. In endometriotic tissues, a high-estrogen environment associated with up-regulation of the aromatase gene has been well documented. There is accumulating evidence supporting a concept that endometriosis is a disease associated with an epigenetic disorder. Epigenetics is one of the most expanding fields in the current biomedical research. The word ‘epigenetics’ refers to the study of mitotically and/or meiotically heritable changes in gene expression that occur without changes in the DNA sequence. The disruption of such changes (epigenetic aberration or disorder) underlies a wide variety of pathologies. Epigenetic regulation includes DNA methylation and histone modifications, and is responsible for a number of gene transcription associated with chromatin modifications that distinguish the states of diseases. In this review, we summarized our studies as well as recent studies from other laboratories using an epigenetic approach focused on DNA methylation. We also summarized studies using advanced technologies including Genome-Wide (GW) methylation profiling analysis and GW Association Study (GWAS). We reviewed recent monozygotic twins studies in relation to environmental factors since they may provide insight into the epigenetic background of endometriosis. Finally, we referred to a new concept of GW DNA methylation.

2. INTRODUCTION

Endometriosis, a common benign gynecologic disorder, is an estrogen-dependent and inflammatory disease characterized by the presence of endometrium-like tissues primarily on the pelvic peritoneum and ovaries (1). Its prevalence in approximately 10% of women of reproductive age adversely affects their quality of life because of chronic pelvic pain and infertility (1). Approximately 40% of infertile women have endometriosis (2), and approximately 1% of endometriotic tissues progresses to malignancy (3, 4).

Epigenetics is one of the most promising and expanding fields in the current biomedical research. The word ‘epigenetics’ refers to the study of mitotically and/or meiotically heritable changes in gene expression that occur without changes in the DNA sequence (5). The disruption of such changes (epigenetic aberration or disorder) underlies a wide variety of pathologies including cancer (6,7). Epigenetic regulation includes DNA methylation and histone modifications (8,9), and is responsible for a number of gene transcription associated with chromatin modifications that distinguish the various cell types and the states of diseases. Cancer and many other diseases show aberrant epigenetic regulation (10). In terms of DNA methylation, cancer cells show GW hypomethylation and site-specific hypermethylation of promoter CpG islands (6).

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This may lead to transcriptional silencing and aberrant transcription from incorrect transcription start sites (8). In addition, a recent study comparing colorectal cancer tissue with its normal counterpart suggests changes at the CpG island shores (11). In normal cells, CpG islands and CpG island shores are under the control of physiological methylation, allowing normal gene transcription.

In this review, we include previous epigenetic studies on endometriosis (12, 13). As suggested by Guo (12), there is accumulating evidence supporting a concept that endometriosis is an epigenetic disease. A concept of DNA methylation in endometriosis is changing and widely expanding as anticipated (13). Here we describe how the field of epigenetics is reshaping the current thinking about endometriosis. As introduction, we summarize our recent studies on aberrant aromatase expression in endometriosis from the viewpoint of epigenetic disorder, and describe recent advances in endometriosis research using the epigenetic approach focused on DNA methylation. In addition, we describe the advanced technology of Genome-Wide (GW) methylation analysis and GW association study (GWAS). Finally, we summarize recent studies on epigenetic differences in monozygotic twins and environmental factors, since they may provide insight into the epigenetic background of endometriosis.

3. ESTROGEN AND ENDOMETRIOSIS

Endometriosis, a common benign gynecological disorder, is characterized by the presence of endometrial-like tissue outside the uterine cavity (1). Although the pathogenesis of endometriosis remains controversial, the retrograde menstruation theory by Sampson is widely accepted (14). Retrograde menstruation has been shown in most menstruating women, but an important question is why only some women develop endometriosis. The answer seems to come from a number of factors that facilitate the implantation and the propagation of endometriotic tissue. In the endometriotic tissue, immune cells, including macrophages, lymphocytes, NK cells, mast cells, and dendritic cells (15, 16, 17, 18), have been suggested to play pivotal roles through cytokines and growth factors in the pathophysiology of endometriosis (19, 20, 21, 22). Several lines of experimental data suggest these roles. First, the endometrial cells in retrograde menstruation must escape from apoptotic cell death to survive. Supporting this concept, we demonstrated the apoptosis-resistant phenotype of endometriotic cells (23), and then identified survivin as an anti-apoptotic molecule (24).

Along with these findings, we observed that peritoneal fluid levels of IL-8 significantly enhanced proliferation of endometriotic cells (25), suggesting that IL-8 may promote the progression of endometriosis. Along with these findings, we observed that peritoneal fluid levels of IL-8 significantly enhanced proliferation of endometriotic cells (25), suggesting that IL-8 may promote the progression of endometriosis. In addition, peritoneal fluid levels of TNF α induced the expression of IL-8 in endometriotic cells (26). We then compared the expression of IL-8 in endometriotic cells of patients treated with

gonadotropin-releasing hormone (GnRH) agonist and those of patients without treatment. Interestingly, estrogen enhanced TNF α -induced IL-8 expression in endometriotic cells from patients without GnRH agonist treatment, while in endometriotic cells from GnRH agonist-treated patients, estrogen and TNF α did not show any significant effect (27). These observations suggest that the reduced estrogen environment associated with decreased cytokine production is a pharmacologic effect of GnRH agonist-treatment in endometriosis.

3.1. Pathophysiologic background of a high estrogen environment in endometriosis

Endometriosis commonly occurs in reproductive-age women and rarely in post-menopausal women. Endometriotic tissue growth depends on ovarian steroids, thus medical treatments aim to reduce ovarian steroidogenesis. Continuous exposure to GnRH agonist results in desensitization or down regulation of GnRH receptors leading to reduced serum gonadotropin levels and reduced ovarian hormone production. Treating endometriosis with GnRH agonist reduces both the number of observable endometriotic implants and the frequency and severity of associated pain (28, 29). Likewise, inhibition of estrogen production by progestins or aromatase inhibitors reduces endometriotic lesions and clinical symptoms (30). These observations support the concept that endometriosis is an estrogen-dependent inflammatory disease (1).

Three major sites for estrogen production are recognized in women with endometriosis: (a) *de novo* synthesis in the ovary, (b) an intrinsic system that depends on aromatase, which converts circulating androstendione to estradiol (*intracrine*) in skin and adipose tissue, and (c) a *de novo* and an *intracrine* systems in endometriotic tissues (31, 32). Among these sites, local estrogen production, which depends on aberrantly expressed aromatase in endometriotic implants, plays an important role in the pathophysiology of endometriosis (33, 34, 35). Local estrogen production by these implants may contribute to the progression of endometriosis even under the hypoestrogenic environment produced by GnRH agonist exposure (35).

3.2. Aberrant aromatase expression in endometriosis

Aromatase, an enzyme that catalyzes the conversion of androgens to estrogens, is a key molecule for estrogen production. Aromatase is encoded by a single copy gene *CYP19* on chromosome 15q21. *CYP19* expression is regulated in a tissue-specific manner, in which alternative usage of multiple promoters with each unique *cis*-acting element has been known (36). Therefore, identifying the promoter usage becomes the first step in understanding the molecular background of aromatase expression in specific tissues. Bulun *et al.* previously reported that promoter II is the most potent promoter functioning in endometriotic cells from endometrioma (35). In addition to promoter II, we recently demonstrated that two proximal promoters, I.3 and I.6, are used additionally in endometriotic cells (32). At the same time, we observed that aromatase transcription in endometrial cells was at a

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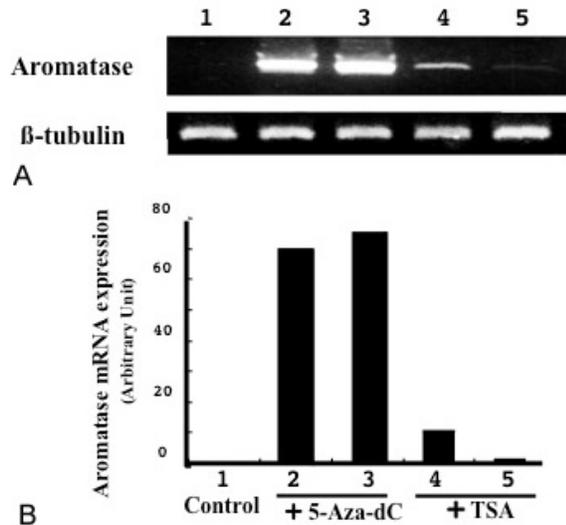


Figure 1. Effect of 5-aza-deoxycytidine(5-Aza-dC)-treatment on aromatase mRNA expression in endometrial cells. Endometrial cells were treated with either 5-Aza-dC for 96 hours or Trichostatin A (TSA) for 24 hours. A representative RT-PCR result: lane 1, untreated control, lanes 2 and 3, treated with 5-Aza-dC, and lanes 4 and 5, treated with TSA. B. Results from semi-quantitative analysis.

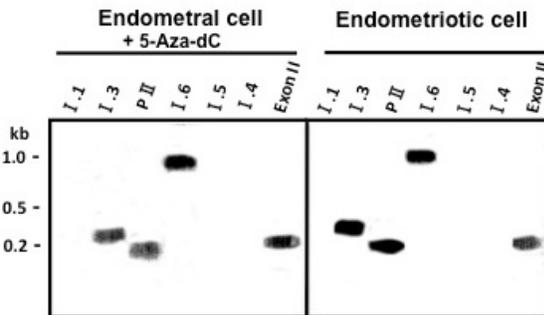


Figure 2. Promoter usage of aromatase mRNA expression in 5-Aza-dC-treated endometrial cells. Results from Exon I-specific RT-PCR. Left: 5-Aza-dC-treated endometrial cells, Right: endometriotic cells.

marginal level depending on the same 3 promoters as those of endometriotic cells (32). From these observations, we hypothesized that the up-regulation of aromatase gene in endometriotic cells may be an epigenetic disorder, since hypermethylation of promoter region in tumor suppressor gene associated with gene silencing has been known.

3.3. DNA demethylation and aromatase mRNA induction in endometrial cells

We hypothesized that an epigenetic disorder may lead to the up-regulation of aromatase mRNA expression in endometriotic cells. We challenged this hypothesis: after treating endometrial cells with 5-aza-deoxycytidine (5-Aza-dC, competitive inhibitor for DNA methyltransferase) for

96 hours, aromatase transcription was markedly up-regulated in the cells (Figure 1) (32). This is the first demonstration that epigenetic modification enhances aromatase mRNA expression. It is important to note that the enhanced aromatase mRNA expression was dependent on the same promoters as those in endometriotic cells (Figure 2). In similar experiments where the effect of Tricostatin A (TSA, histone deacetylase inhibitor with a wide spectrum of substrate specificity), instead of 5-Aza-dC, on aromatase mRNA expression was examined, we observed little effect, suggesting that one of the major factors that suppresses aromatase mRNA expression in endometrial cells is the methylation of the aromatase gene and/or its *trans*-acting factor gene. Alternatively, the observation suggests that a disorder (*i.e.* demethylation) of a putative methylation-dependent suppression mechanism may lead to the up-regulation of aromatase mRNA expression in endometriotic cells.

3.4. Demonstration of hypomethylated CpG island within the aromatase gene in endometriotic cells

We searched for the unmethylated CpG locus within the aromatase gene in endometriotic cells (37). We predicted a CpG island at approximately 20 kb upstream from the end of Exon II. In endometriotic cells, the CpG sequence was hypomethylated, while in endometrial cells, the upstream half was hypermethylated and recognized by the methyl-CpG binding proteins, MBD1 and MeCP2 (37). The downstream half was hypomethylated in both endometrial and endometriotic cells. Because the CpG sequence is located at the promoter-distal region, it is tempting to speculate that the sequence may act as a *cis*-acting element under the control of methylation. Our preliminary study shows that the CpG sequence tagged to a reporter gene behaves as an enhancer when introduced into cells (Izawa M, unpublished observation).

4. NUCLEAR RECEPTORS AND EPIGENETIC ABERRATION IN ENDOMETRIOSIS

Endometriosis is an estrogen-dependent disease. In addition to the hormonal level in endometriotic tissue, the estrogen receptor (ER) plays pivotal roles in the pathogenesis and progression of endometriosis. Earlier studies have focused on the expression of ER α as well as ER β in the eutopic endometrium and in endometriotic lesions. Simultaneous expression of ER α and ER β indicates that the estrogen action might be transmitted in a cooperative manner (38, 39). In contrast to the high ER α /ER β expression ratio in the eutopic endometrium, a lower expression of ER α and a markedly higher expression of ER β in ovarian endometriomas have been reported (40, 41). The higher ER β expression in endometriotic tissue may depend on the hypomethylation of the ER β -promoter region (42). The increased ER β expression in endometriotic tissues may suppress ER α expression (43). However, in addition to a wild type ER β , we recently observed multiple ER β splice variants in endometriotic cells (Izawa M, unpublished observation). Additionally, ER β variants were observed in the endometrium with or without endometriosis (44). Therefore, ER system in endometriosis should be carefully re-evaluated.

4.1. ER β gene: hypomethylation and up-regulation

That the development and progression of endometriosis depends on estrogen is well known (45, 46). ER α and ER β function as transcription factors and are believed to play key roles in tissue growth of endometrium and endometrioma (40, 41). Previous studies demonstrated markedly higher levels of ER β and lower levels of ER α in endometriotic tissues and endometriotic stromal cells (39, 40, 41). Differences in the ER α /ER β ratio between endometriotic and endometrial stromal cells are suggested to have important functional implications (42). Clarifying the molecular basis of the estrogen receptor system becomes an important tool in understanding the pathophysiology of estrogen action in endometriosis. Recently, Xue *et al.* (42) demonstrated that the ER β promoter is hypomethylated in endometriotic cells. Hypomethylation caused the higher expression of ER β gene in endometriotic cells, while hypermethylation silenced the expression in endometrial cells. Treatment with a demethylating agent significantly increased ER β mRNA expression in endometriotic cells. They proposed that enhanced ER β transcription in endometriotic cells takes over the ER α promoter activity, thus favoring the suppression of ER α levels (43). The increased ER β /ER α ratio in endometriotic cells may lead to increase ER β binding to the promoter of the progesterone receptor gene leading to the down-regulation. It is important to note that five ER β isoforms have been demonstrated recently in several cell types (47). That finding prompts us to re-evaluate the ER β expression in endometriosis.

4.2. Progesterone receptor (PR) gene: hypermethylation and down-regulation

Progesterone induces differentiation of endometrial stromal cells to decidualized cells and glandular epithelial cells to the secretory phenotype. Representative molecular markers of progesterone action include increased production of epithelial glycodefin and stromal prolactin in the endometrium (48, 49). Progesterone, however, induces lower levels of prolactin expression in endometriotic cells than in endometrial stromal cells, suggesting progesterone resistance in endometriosis (41). Progesterone resistance is known as a feature in some endometriosis and can be attributed to the low level of progesterone receptor (PR) in endometriotic tissue (1, 50, 51, 52). PR-A and PR-B forms are expressed in the stromal and epithelial components of the endometrium (52). In the endometrium, expressions of PR-B and PR-A are progressively up regulated during the proliferative phase to their highest level immediately before ovulation, and diminish thereafter, suggesting that estradiol stimulates PR expression (43). Reportedly, PR-B is at a marginal level and PR-A is downregulated in endometriotic tissues (52, 53, 54). PR level may be related to the less responsiveness to progesterone in some patients (54, 55). Wu *et al.* (56) have shown that the promoter region of PR-B is hypermethylated in endometriosis, which may lead to the PR-B down-regulation. They recently showed that prolonged stimulation of TNF- α induced partial methylation in the promoter region of PR-B associated with decreased expression of PR-B in an immortalized epithelial-like endometriotic cell line (57). This seems to

provide evidence that phenotypic changes in endometriosis, such as chronic inflammation and increased production of pro-inflammatory cytokines, may cause epigenetic aberrations leading to changes in gene expression (58).

5. GENES UNDER EPIGENETIC ABERRATION IN ENDOMETRIOSIS

5.1. Steroidogenic factor-1 (SF-1) gene: hypomethylation and up-regulation

Steroidogenic factor-1 (SF-1) is a transcriptional factor essential for activation of multiple steroidogenic genes for estrogen biosynthesis, such as steroid acute regulatory (StAR) and aromatase (59, 60, 61). SF-1 is usually undetectable in eutopic endometrial cells (53). It has been demonstrated that SF-1 mRNA and protein levels in endometriotic cells were significantly higher than those in eutopic endometrial cells (53, 61). Xue *et al.* identified a classical CpG island at the promoter region of the SF-1 gene and showed that the SF-1 promoter has increased methylation in eutopic endometrial cells (53). In endometrial cells, the silencer-type transcription factor MBD2 is recruited to the methylated SF-1 promoter, and prevents its interaction with transcriptional activators, resulting in silencing of the SF-1 gene. On the other hand, the SF-1 promoter is hypomethylated in endometriotic cells. SF-2, a transcription factor highly expressed in endometriotic tissues, binds to the unmethylated SF-1 promoter and activates its transcription in endometriotic cells (62). The SF-1 expression is under epigenetic control that permits the binding of activator complexes to the SF-1 promoter (53, 62). SF-1 expression in endometriosis may enhance aromatase expression leading to local estrogen production. Treatment with a demethylation agent has been shown to increase SF-1 mRNA levels in eutopic endometrial cells (45).

5.2. E-cadherin gene: hypermethylation and down-regulation

Down-regulation of E-cadherin, a known metastasis-suppressor protein in epithelial tumor cells (63), has been shown in endometriotic cells (64). In two immortalized endometriotic cell lines, the E-cadherin gene was found to be hypermethylated at the promoter region, and treatment with a histone deacetylase inhibitor, trichostatin A, induced expression (65). Interestingly, the increased promoter methylation of the E-cadherin gene during aging has been demonstrated (66).

5.3. HOXA10 gene: hypermethylation and down-regulation

HOXA10 has been expressed in the endometrium, and its expression is under the control of estrogen and progesterone (66, 67, 68). The roles in endometrial development during the menstrual cycle and in establishing uterine receptivity have been suggested (67, 68). In women with endometriosis, HOXA10 expression is significantly decreased in the eutopic endometrium during the secretory phase, indicating functional defects in uterine receptivity (67, 69). The promoter region of HOXA10 gene was found to be hypermethylated in the eutopic endometrium from women with endometriosis (70). As

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promoter hypermethylation has been suggested as an epigenetic marker of gene silencing, the promoter hypermethylation may be related to the HOXA10 down-regulation in the eutopic endometrium of women with endometriosis (67). PR down-regulation may be related to the decreased expression of the HOXA10 gene.

6. GENOME-WIDE (GW) PROFILING OF DNA METHYLATION AND GW ASSOCIATION STUDY (GWAS) IN ENDOMETRIOSIS

Methylation of DNA provides a layer of epigenetic controls that has important implications for disease including endometriosis. There has been a revolution in DNA methylation analysis technology. Analyses can now be performed on a genome-scale and entire methylomes can be characterized at single-base-pair resolution (71). In endometriosis, a number of aberrant gene expressions have been demonstrated (72, 73). These aberrations may be related to aberrant DNA methylations. It is unknown whether global alterations in DNA methylation patterns occur in endometriosis and to what extent they are involved in its pathogenesis. A whole-genome scanning of methylation status in more than 25,000 promoters, using methylated DNA immunoprecipitation with hybridization to promoter microarrays has been conducted for the first time (74). The results showed that, in line with the current theory of the endometrial origin of endometriosis, the overall methylation profile was highly similar between the endometrium and the endometriotic lesions. In addition to promoter CpGs, a number of promoter distal CpG sequences are known. We recently challenged a GW profiling of DNA methylation in more than 450,000 CpG sites in endometriotic and endometrial cells, and identified more than 1,000 CpGs differentially methylated not only in the promoter region, but in promoter distal CpG islands (Izawa M, unpublished observation).

Through a GWAS and a replication study using a total of 1,907 Japanese individuals with endometriosis and 5,292 controls, a significant association of endometriosis with rs10965235 ($P = 5.57 \times 10^{-12}$), odds ratio = 1.44), which is located in CDKN2BAS on chromosome 9p21, encoding the cyclin-dependent kinase inhibitor 2B antisense RNA was identified (75). The findings suggest that these regions are susceptible loci for endometriosis. A GWAS was performed using two case-control cohorts genotyped with the Affymetrix Mapping 500K Array or Genome-Wide Human SNP Array 6.0 in Japanese women with endometriosis (76). A GWAS in 3,194 individuals with surgically confirmed endometriosis and 7,060 controls from Australia and the UK was conducted (77). The strongest association signal was reported on 7p15.2 (rs12700667) for endometriosis. rs12700667 is located in an intergenic region upstream of the plausible candidate genes NFE2L3 and HOXA10.

7. EPIGENETIC ABERRATIONS AND ENVIRONMENTAL FACTORS IN ENDOMETRIOSIS: A LESSON FROM MONOZYGOTIC TWINS STUDIES

The known epigenetic modifications are DNA methylation and histone modifications, including

methylation, acetylation, ubiquitylation and phosphorylation (78). The functional and biological significance of the epigenetic alterations accumulate over a life span. The earliest studies found a pattern of low global DNA methylation levels in aged mammalian tissues (79). A recent monozygotic (MZ) twins study showed a line of evidence that epigenetic variants accumulate during aging independently of the genetic sequence (80). The study tested the epigenetic contribution to twin discordance and elucidated the effect of environmental characteristics on gene function. The results revealed that epigenetic difference between siblings is associated with phenotypic discordance, which might be attributed to an unshared environment.

7.1. Epigenetic difference and environmental factors

The association between environmental factors and phenotypic discordance within MZ twins has been noticed. However, little was known about the molecular basis by which environmental factors influence gene functions (81). Typical examples are the abnormal intrauterine environment associated with epigenetic down-regulation of genes (82, 83) and the maternal diet associated with the DNA methylation profile of offspring (84, 85, 86). Gene function and chromatin structure can be modulated by environmental factors (87, 88). In response to a methyl-deficient diet, a significant decrease in repressive dimethyl-H3K9 associated with the up-regulation of targeted gene was demonstrated in mice (89). Environmental factors, including endocrine disrupting chemicals, may affect the epigenome leading to the onset of endometriosis *in utero*. There might be epigenetic changes during ontogenic development (90, 91).

7.2. DNA methylation

The great fidelity with which DNA methylation patterns in mammals are inherited after each cell division is ensured by the DNA methyltransferases (DNMTs). However, the aging cell undergoes a DNA methylation drift. Early studies showed that global DNA methylation decreases during aging in many tissue types (79). The loss of global DNA methylation during aging is probably mainly the result of the passive demethylation of DNA as a consequence of a progressive loss of DNMT activity (92). Several specific regions of the genomic DNA become hypermethylated during aging (93). Methylation of promoter CpG islands in nontumorigenic tissues has been reported for several genes, including ER (93). Interestingly, genes with increased promoter methylation during aging include the E-cadherin gene (66), which is down-regulated and hypermethylated in endometriotic cells (64).

7.3. Histone modification

Histone modifications have a defined profile during aging. For example, the trimethylation of H4-K20, which is enriched in differentiated cells (94), increases with age (95, 96), and decreases in cancer cells (96, 97, 98, 99, 100). A decrease in the histone trimethylation has been observed in the liver after the long-term treatment with the hepatocarcinogen tamoxifen (97). The loss of trimethylated H4-K20 in cancer can be caused by the loss of expression of the H4-K20-specific methyltransferase Suv4-20h (90).

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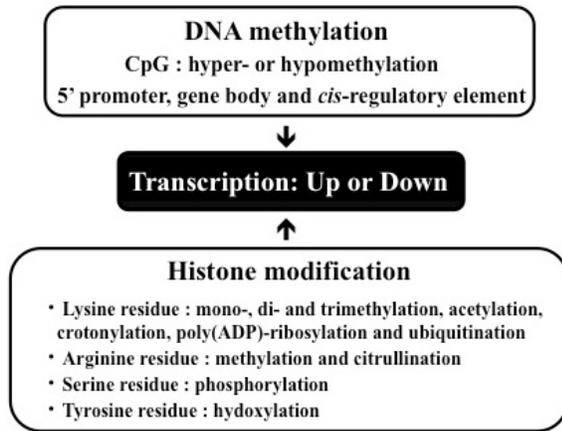


Figure 3. Epigenetic regulation includes DNA methylation and histone modification. It is responsible for a number of gene transcription associated with chromatin modifications. Once disordered, it may lead to an aberrant gene expression.

Although approximately 100 histone methyltransferases and demethylases have been identified in human genome, only a subset of histone methyltransferase inhibitor is in clinical trials for cancer treatment (102). Aging is the risk factor associated with cancer development (103). The down-regulation of genes preventing the aging process may be in cancer cells, too. Although a previous study concluded that age-related differences in CpG island DNA methylation might not be apparent (104), the advanced human epigenome projects (71, 105) may provide insight into understanding epigenetic aberrations in endometriosis.

8. CONCLUSION

Epigenetics is one of the most promising and expanding fields in the current biomedical research of diseases including endometriosis. Most important is its translational application. One of immediate questions to be clarified in endometriosis is which genomic sequence undergoes DNA hypermethylation or hypomethylation? Here we focused on DNA methylation and reviewed current literatures in endometriosis.

Aberrant methylation of CpG islands in 5' promoters has been suggested to be associated with transcriptional silencing or up-regulation in endometriosis (42, 53, 56, 70). Recent GW methylation studies suggest that tissue- and cell type-specific methylation is present only in a small percentage of CpG islands in 5' promoters, while a far greater proportion of CpG island methylation is across gene bodies (106, 107). In addition, functionally different types of DNA modification, methylation and hydroxymethylation, have been identified (106, 108). Therefore, methylation of CpG islands in 5' promoters alone may not be a major player in the aberrant gene expression.

Epigenetics is currently expanding from DNA methylation to histone modifications (Figure 3). The

development of high-throughput technologies associated with a new concept of DNA methylation may accelerate the study of epigenetic aberration in endometriosis. Using the epigenetic concept as the lens, new diagnostic markers or therapies may be developed to overcome serious problems in patients with endometriosis.

9. ACKNOWLEDGEMENT

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