Aberrant endometrial DNA methylome of homeobox A10 and catechol-*O*-methyltransferase in endometriosis

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Received: 14 September 2016 / Accepted: 12 December 2016 / Published online: 10 January 2017
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Abstract

Purpose Differential methylation of both *HOXA10* and catechol-*O*-methyltransferase (*COMT*) has been reported in different endometrium disorders, and the two genes are linked through the estrogen pathway. The current study investigates the DNA methylation of *HOXA10* and *COMT* in ectopic and eutopic endometrial tissues and its correlation with and the occurrence of endometriosis in women from Xinjiang province in China.

Methods In the current study, 120 patients with endometriosis were recruited from our hospital between January 2011 and June 2014. The DNA methylation sites of *HOXA10* and *COMT* were detected using a DNA methylation array. The methylation levels of specific sites were compared between ectopic and eutopic endometrial tissues via pyrosequencing.

Results Five differentially expressed CpGs were localized in the promoter region of the *COMT* gene and expressed significantly higher in the ectopic endometrium than the eutopic endometrium ($P < 0.001$). Two out of the five

differentially expressed CpGs in the *HOXA10* gene located in the promoter region were both significantly lower (nearly half) in the ectopic endometrium than the eutopic endometrium ($P < 0.001$).

Conclusions To summarize, significant differential methylation of *HOXA10* and *COMT* promoter regions was found between the ectopic and eutopic endometrial tissues. This is the first study investigating the methylation of *HOXA10* and *COMT* genes and their linkage to endometriosis in Chinese patients.

Keywords Endometriosis · *HOXA10* · Catechol-*O*-methyltransferase · DNA methylation

Introduction

Endometriosis affects up to 15% women almost exclusively in the reproductive age, causing infertility and development of

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adhesions due to the extra-uterine growth of endometrium-like tissue [1, 2]. The molecular cause of endometriosis is multifactorial, including disease occurrence and severity influenced by genetic, environmental, and lifestyle factors [3]. The existing theories on the cellular origin of endometriosis include retrograde menstruation, coelomic metaplasia, embryonic cell rest theory, and stem cell theory [4, 5]. Among these, Sampson's retrograde menstruation model, retrograde flow of sloughed endometrial cells/debris via the fallopian tubes into the pelvic cavity during menstruation, remains most widely accepted [6]. Although endometriosis is a benign disease, increasing pieces of evidence support the hypothesis that it represents the initial stages of neoplastic processes. The common characteristics shared by endometriosis and cancer include the ability to evade apoptosis, the adult stem cell-like dysregulation, neovascularization, progressive course, implantation at distant sites, the creation of a microenvironment that renders its course independent, and mobilization of the immune system [7].

Aberrant DNA methylation has been identified as a vital mechanism in tumorigenesis and multiple other diseases [8, 9]. A recent study analyzed the genome-wide DNA methylation in patients with endometriosis and identified 42,248 differentially methylated CpGs in the endometriosis cells as compared to the healthy cells [10]. The homeobox gene (HOX) clusters were some of the maximum differentially methylated families of genes [10]. HOX genes are dynamically expressed in the endometrium, where they are necessary for endometrial growth, differentiation, and implantation. *HOXA10* gene hyper-methylation has recently been demonstrated to silence the gene expression and accounts for the decreased expression in the women with endometriosis [11, 12]. Catechol-*O*-methyltransferase (*COMT*) can inactivate catechol estrogens, metabolites of estradiol (E2) that can bind estrogen receptor, and these catechols are involved in the pathogenesis of endometriosis [13]. There are increased levels of membrane-bound *COMT* protein in ovarian endometrial tissues [13], but the relevance of *COMT* polymorphisms to endometriosis remains in debate [14, 15], and the methylation status of *COMT* in endometriosis remains to be revealed. Because endometriosis is an estrogen-dependent disease, and *COMT* and *HOXA10* are upstream and downstream of estrogen regulation, respectively [16, 17], they can be critical components in the pathogenesis of endometriosis as well as its potential progression to cancer. We present a simplified schematic of their possible roles in the pathogenesis of endometriosis in Fig. 1.

Hitherto, most studies encompassing the epigenetic causes of endometriosis or endometrial cancers are conducted in the Western world. Little has been investigated in China. The current study recruited patients who were long-term residents of Xinjiang province. We aimed to investigate the DNA

Fig. 1 Simplified schematic of the potential roles of *HOXA10* and *COMT* in the pathogenesis of endometriosis. *HOXA10* acts downstream of activated estrogen receptor (ER); in endometriosis, it is hyper-methylated and this action is decreased. *COMT* degrades 2-hydroxyestradiol (2-OHE2) the product of 2-hydroxylation of estradiol (E2) and thus decreases the level of available E2 for ER binding and translocation to the nucleus

methylation of *HOXA10* and *COMT* in ectopic and eutopic endometrial tissues and its correlation with and the occurrence of endometriosis.

Materials and methods

Patients

The present study recruited patients between January 2011 and June 2014 at the Department of Gynecology, The First Affiliated Hospital of Xinjiang Medical University from the southern, northern, and eastern Xinjiang province. The patients who fulfilled the following criteria were included: (1) provided full consent; (2) were of childbearing age (between 20 and 48 years old); (3) were scheduled to receive laparoscopic or open abdominal surgery for treating endometriosis as primary indication; (4) did not have abnormal menstrual cycle history; (5) did not receive hormonal treatment during the past 3 months; and (6) residing for more than 5 years in Xinjiang province with no inter-racial marriage in the past three generations. Patients excluded were those who (1) had gestational diseases and impaired uterus and, thus, could not allow the sample collection of eutopic endometrial tissues; (2) had cardiovascular, neural, lung, liver, or kidney diseases; and (3) had hypertension, diabetes, cancer, or chronic infectious diseases. The study cohort consisted of 60 endometriosis patients and 120 endometrial tissues (ectopic and eutopic, 60 of each). Information about the patients' demographics, tissues, and disease stage (according to the revised American Fertility

Table 1 Primers used in PCR amplifications and sequencing

Region	Primer sequences	Product length (bp)	Sequencing primers
COMT1	Forward: TGTATTTTAGTTTGGGCGATAGAGTA Reverse: CTAATAATTTTATAATTCTAAATAAACTCTAAAA	109	AAAAAAAAGTTTGGGATT
COMT2	Forward: GAATTATAAAATTATTAGAAGTAATGTTTGT Reverse: CGTATTATCTCCACTCTACCTACTCATAC	123	GTAATGTTTGTGTTTTATTGTTGT
COMT3	Forward: TGTGATTTTCGTATGAGTAGGTAGAGTG Reverse: CTAAATCTAAATAAAAACCACCATCC	115	AGTAGGTAGAGTGGAGATAA
HOXA10	Forward: TGGAAAGAGTTAGGGAAATGTTTAGAG Reverse: ACCTAACTTTCCCAAATAAAATCCC	73	AGGGAAATGTTTAGAGGTA

Society [rAFS] classification) was assimilated. The study was conducted under the regulation of the research ethics committee of the Department of Gynecology, The First Affiliated Hospital of Xinjiang Medical University.

DNA extraction

Both ectopic and eutopic endometrial tissues were collected from the participants during the surgery. One gram of each tissue sample was immediately stored in liquid nitrogen and then at -80°C until further usage. The DNA from the endometrium tissues was extracted using the DNA extraction kit (QIAGEN, CA, USA) following the manufacturer's protocol.

DNA methylation array

Bisulfite conversion was performed after DNA extraction. DNA methylation array was then conducted using the Infinium Human Methylation 450 BeadChip array (Illumina, CA, USA) based on the manufacturer's protocol. Briefly, the genome DNA was first treated with sodium bisulfite and then used for the PCR amplification of *HOXA10* and *COMT*. PCR primers (Primer-G) are listed in Table 1. Subsequently, the PCR products were treated with shrimp alkaline phosphatase (SAP) and the resulting fragments analyzed to detect the differentially methylated CpG sites (selection criteria: false discovery rate (FDR) <0.05 and beta change ≥ 0.2). The differentially methylated sites were further confirmed with *HOXA10*

or *COMT* gene sequence (NCBI) and those located in the promoter region were identified.

Methylation-specific PCR

To assess the methylation status of the *HOXA10* and *COMT* promoter regions, we employed methylation-specific PCR using primers for differentially methylated regions. PCR primers and conditions are listed in Tables 1 and 2, respectively. Each methylation-specific PCR reaction utilized 25 ng of sodium bisulfite to convert the DNA (Zymo EZ DNA Methylation kit, ZYMO Research, CA, USA) from the template of either ectopic and eutopic endometrial tissues. The female genomic DNA that was either fully methylated and bisulfite converted or fully demethylated and bisulfite converted was used as a control.

The PCR products were resolved on 3% agarose gel and visualized with ethidium bromide under ultraviolet (UV) light. If the primers targeting the methylated sequence resulted in successful PCR amplification, while the primers targeting the unmethylated sequence failed to result in PCR amplification, this suggested that homozygotic methylation was present in the 5' CpG of the promoter. If the specific primers targeting both the methylated and unmethylated sequences both resulted in PCR amplification, this suggested that heterozygotic methylation had occurred in the promoter region. If the primers targeting the methylated sequence failed to initiate PCR amplification, this indicated the absence of methylation in the promoter region.

Table 2 PCR conditions for pyrosequencing assays

	COMT1		COMT2		COMT3		HOXA10	
	Temperature	Time	Temperature	Time	Temperature	Time	Temperature	Time
Predenaturation	95 $^{\circ}\text{C}$	3 min	95 $^{\circ}\text{C}$	3 min	95 $^{\circ}\text{C}$	3 min	95 $^{\circ}\text{C}$	3 min
Denaturation	94 $^{\circ}\text{C}$	25 s	94 $^{\circ}\text{C}$	25 s	94 $^{\circ}\text{C}$	25 s	94 $^{\circ}\text{C}$	25 s
Annealing	58 $^{\circ}\text{C}$	25 s	56 $^{\circ}\text{C}$	25 s	55 $^{\circ}\text{C}$	25 s	58 $^{\circ}\text{C}$	20s
Extension	72 $^{\circ}\text{C}$	25 s	72 $^{\circ}\text{C}$	25 s	72 $^{\circ}\text{C}$	25 s	72 $^{\circ}\text{C}$	20s
Repair extended	72 $^{\circ}\text{C}$	5 min	72 $^{\circ}\text{C}$	5 min	72 $^{\circ}\text{C}$	5 min	72 $^{\circ}\text{C}$	5 min
Cycling	35		35		35		35	

Table 3 Patients' demographics and disease stages

Race		Han (<i>N</i> = 30)	Uyghur (<i>N</i> = 30)
Age (years old)	Range	20–48	
	Mean	41	42
Tissue (<i>n</i>)	Ectopic endometrium	30	30
	Eutopic endometrium	30	30
Disease stage (<i>n</i>)	I–II	10	10
	III–IV	20	20

Pyrosequencing

A pyrosequencing assay was used to analyze the methylation levels of each CpG site in the promoter region of *HOXA10* and *COMT* through the PyroMark Q96 ID system (QIAGEN). A forward primer with the M13 sequence at the 5' site of one of the previous methylation-specific PCR primers (Primer M) was designed for either the *HOXA10* or *COMT* promoter region with Assay Design Software 1.0 (Biotage, MA, USA) (Table 1). The PCR reaction was carried out using Primer M and the amplicons of the previous methylation-specific PCR reactions that showed homozygotic or heterozygotic methylation of the promoter region. After confirming the anticipated amplifications using DNA electrophoresis, the PCR amplification of the pyrosequencing template was carried out using the biotinylated M13 primer and PCR products of the previous reaction. The biotinylated PCR products were then immobilized onto the streptavidin beads, and the pyrosequencing reaction was conducted by following the system protocol. Pyrograms were produced in the CpG methylation analysis software PyroMark CpG SW 1.0.11 (QIAGEN), and sequencing results, including sequence and methylation level, were compared between the ectopic and eutopic endometrial tissues based on the methylation sites of *HOXA10* and *COMT*, respectively.

Statistical analysis

Data were analyzed using the R software version 2.15.2 (<https://www.r-project.org/>). Results are presented as mean $\pm$ standard deviation (SD). Paired Student's *t* test was used to compare the results between ectopic and eutopic endometrium samples. Two-tailed *P* < 0.05 was considered statistically significant.

Results

Subjects

In the present study, we enrolled 60 endometriosis patients from south, north, and east of Xinjiang province between January 2011 and June 2014 at the Department of Gynecology, The First Affiliated Hospital of Xinjiang

Medical University. These 60 patients constituted of 30 from the Han population and the other 30 from Uyghur. The mean ages of these patients were 41 and 42 years (Han and Uyghur, respectively; range from 20 to 48 years). Both the ectopic and eutopic endometrial tissue samples were obtained from each patient, as well as another clinical information, such as disease stage of the patients as summarized in Table 3.

Differential methylation sites of *HOXA10* and *COMT*

Based on the DNA methylation array, five differentially methylated CpG sites were detected each in the *COMT* and *HOXA10* genes (Table 3). All the five CpG sites in *COMT* were located in the promoter region, while only two out of the five CpGs in the *HOXA10* gene were located in the promoter region (*CpG sites in Table 4).

Methylation levels of promoter region CpGs

Based on the pyrosequencing results, the methylation levels of the five differentially methylated CpG sites in *COMT* promoter region were significantly higher in the ectopic endometrium than the eutopic tissue (Fig. 2,

Table 4 Differential methylation sites in *COMT* and *HOXA10*

Gene	Methylation sites
COMT	cg08289189 ^a
	cg03724721 ^a
	cg12728623 ^a
	cg06787004 ^a
	cg18773129
HOXA10	cg25836061 ^a
	cg15864691
	cg09636715
	cg08938793
	cg08575233 ^a
	cg05517976 ^a
	cg14935646

^a Differential methylation sites in the promoter region

Fig. 2 Methylation rate of variable methylation sites in the promoter region of *COMT* gene in ectopic and eutopic endometrial tissues. **a** Pyrograms showing pyrosequencing results. **b** Methylation rate of variable methylation sites. *M* ectopic endometrium, *C* eutopic endometrium. ***Between-tissue group comparison $P < 0.001$

$P < 0.001$). Four out of the five sites (except CG18773129) showed nearly double methylation level in the ectopic endometrium than the eutopic endometrium. Contrastingly, the methylation levels of the two differentially methylated CpG sites in *HOXA10* promoter region were significantly lower (nearly half) in the ectopic endometrium than the eutopic tissue (Fig. 3, $P < 0.001$).

Discussion

To our knowledge, this is the first study investigating the methylation of *HOXA10* and *COMT* genes and their linkage to endometriosis in Chinese patients. We found that differential methylation of the *COMT* gene is entirely localized in the promoter region and is strongly upregulated in the ectopic

Fig. 3 Methylation rate of variable methylation sites in the promoter region of *HOXA10* gene in ectopic and eutopic endometrial tissues. **a** Pyrograms showing pyrosequencing results. **b** Methylation rate of variable methylation sites. *M* ectopic endometrium, *C* eutopic endometrium. ***Between-tissue group comparison $P < 0.001$

endometrium. In contrast, the differential methylation of *HOXA10* is not exclusive to the promoter region and the differential methylation of its promoter regions is downregulated in the ectopic endometrium.

Our results are consistent with the previous findings and provide accurate information on the differential methylation of the promoter region of the two genes. Recent animal and human studies have reported *HOXA10* hyper-methylation, a putative mechanism by which *HOXA10* levels could be decreased in endometriosis [11, 12, 18]. In the animal endometriosis models, *Hoxa10/HOXA10* was hyper-methylated at the 5' promoter region and its expression was downregulated in the eutopic endometrium [11, 12]. In humans, three CpG-rich fragments in the *HOXA10* gene were identified. Moreover, *HOXA10* was hyper-methylated on all the fragments in the endometrium of women with endometriosis as compared to the controls [18]. Similarly, we have found hyper-methylation of *HOXA10* promoter in the eutopic endometrium vs. ectopic endometrium, suggesting the downregulation of the gene. However, through the DNA methylation array, we identified only two differentially methylated CpG sites in the *HOXA10* promoter region. This may occur as the current study compared the ectopic and eutopic tissues in endometriosis patients while the previous study by Wu et al. (2005) compared endometrial tissues between endometriosis patients and those who did not have endometriosis [19]. Therefore, the current study provides information on tissue-specific methylation pattern of the *HOXA10* promoter region.

An important and novel finding of this study is the differential methylation of *COMT* in ectopic endometriosis. Hitherto, DNA methylation of *COMT* and its correlation with

endometriosis had not been reported. However, it is understood that *COMT* is likely to play a role in endometriosis [20]. *COMT* inactivates the estradiol metabolites, 2-hydroxy and 4-hydroxy catechols, which have been implicated in the pathogenesis of endometriosis [20]. The CpG methylation density in the membrane-bound *COMT* promoter has been found to be significantly higher in the endometrial cancer tissues than in the adjacent normal tissues [21]. Our finding of hyper-methylation of *COMT* in the ectopic endometrium vs. eutopic endometrial tissues is the first report in endometriosis patients, which suggests a critical role of the *COMT* gene in regulating estrogen-induced malignancies, possibly progressed from endometriosis.

However, the present study has the following limitations. Although the between-tissue group comparison can be satisfied with the current sample size, in order to understand the differential DNA methylation of *HOXA10* and *COMT* by patient disease stage or between the two ethnicities will require a large sample size for future studies. We also did not undertake detailed analysis of the methylation levels of the three other differentially methylated CpG islands that were not located in the promoter region in *HOXA10*; this should be investigated fully later.

Conclusions

To summarize, differential methylation in the promoter region of *HOXA10* and *COMT* genes was found between the ectopic and eutopic endometrial tissues of patients with endometriosis from the Xinjiang province of China. The current study was a

pilot experiment to explore the epigenetic causes of endometriosis in Chinese patients. Our findings indicate that further studies utilizing animal models may provide deeper insight into the molecular mechanisms involving *HOXA10* and *COMT* genes in the development of endometriosis. The racial and disease stage-specific analysis may also further elucidate whether the methylation levels of the two genes are correlated with these factors.

Acknowledgements This study was supported by the National Natural Science Foundation of China (81160077). The funders did not play any role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Compliance with ethical standards The study was conducted under the regulation of the research ethics committee of the Department of Gynecology, The First Affiliated Hospital of Xinjiang Medical University.

Conflict of interest The authors declare that they have no conflict of interests.

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