DNA methylation of HOXA10 in eutopic and ectopic endometrium


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STUDY QUESTION: Does the methylation status of the promoter region of the HOXA10 gene differ in eutopic and ectopic endometrium?

SUMMARY ANSWER: The eutopic endometrium in women with endometriosis is significantly more methylated when compared with controls.

WHAT IS KNOWN ALREADY: Expression of the HOXA10 gene, which is important for successful implantation, is reduced in women affected by endometriosis.

STUDY DESIGN, SIZE AND DURATION: A pilot study was carried out including 18 women admitted for surgery for endometriosis-related pain (cases) and 12 women admitted for surgery because of non-endometriotic disease (control). Sample collection and analysis were performed between November 2010 and July 2013.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Endometrial tissue (eutopic and ectopic) underwent sodium bisulfite DNA modification, PCR amplification of two regions of the HOXA10 promoter and pyrosequencing analysis.

MAIN RESULTS AND THE ROLE OF CHANCE: The eutopic endometrium of women with endometriosis was significantly more methylated compared with endometrium from the control group (sequence 1: 8.68% in cases and 6.25% in the control group: P = 0.037, sequence 2: 11.89% in cases and 9.25% in the control group: P = 0.032). The eutopic endometrium was significantly more methylated than the ectopic tissue in patients with endometriosis (mean difference −3.6 sequence 1: P = 0.001 and −6.0 sequence 2: P = 0.0001).

LIMITATIONS, REASONS FOR CAUTION: The study had a limited sample size and the fertility status of the majority of patients in our study was unknown.

WIDER IMPLICATIONS OF THE FINDINGS: Our data regarding methylation state of the ectopic tissues contribute to a better etiopathologic understanding of endometriosis.

STUDY FUNDING/COMPETING INTEREST(S): No external funding was either sought or obtained for this study. The authors have no conflicts of interests to declare.

Key words: endometriosis / HOXA10 gene / DNA methylation

Introduction

Endometriosis is a common gynecologic disorder with a poorly understood etiopathogenesis. Several theories have been proposed to better understand the etiology of the disease.

The pathophysiology of endometriosis and its impairment of infertility still remains unclear and the most common hypothesis of retrograde menstruation is not sufficient to explain the diverse manifestations in patients. The clinical expression of endometriosis varies considerably from patient to patient and might lead to the belief that the nature of the pathology depends on localization, duration and the patient’s genetic predisposition. It has been assumed that the disease presents different clinical expression based upon individual parameters involving genetic, immunologic and environmental factors and that different types of disease could be variants of the same pathologic process or that they could be caused by different mechanisms. For instance, it has been proposed that rectovaginal, ovarian and peritoneal endometriosis should be considered as three different diseases with three distinct
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Aetiology (Nisolle and Donnez, 1997; Garry, 2004; Bulun, 2009). Studies of the gene expression in ectopic and eutopic endometrium have revealed differences between the various forms of endometriosis (Kwon and Taylor, 2004).

Numerous proposals regarding the nature of the disease have been presented throughout the years; for example, that endometriosis is a hormonal disease, an autoimmune disease, a genetic disease and a disease caused by exposure to environmental toxins, and still after decades of research our understanding of its etiopathogenesis is still inadequate. In recent years, evidence has emerged that endometriosis may be an epigenetic disease (Lu et al., 2013). Different genes are reported to present epigenetic aberrations in endometriosis (D’Hooghe et al., 2003; Wu et al., 2005, 2006; Kim et al., 2007; Xue et al., 2007a,b; Izawa et al., 2008; Lee et al., 2009).

The HOX genes

Homeobox (Hox/HOX) genes encode transcriptional factors involved in embryonic development. Several studies conducted by Taylor et al. regarding the role of these genes in embryogenesis and their expression in humans and mice have demonstrated that hoxa10/hoxa11 are expressed in endometrial glands and stroma throughout the menstrual cycle, with a significantly increased expression during the mid-luteal phase corresponding to the implantation window, an expression of subordinated levels of estrogen and progesterone (Taylor et al., 1997, 1998). Other authors have confirmed the role of hoxa10/hoxa11 in endometrial receptivity in studies on mice (Hsieh-Li et al., 1995; Satokata et al., 1995; Benson et al., 1996) which demonstrated induced infertility due to reduced endometrial receptivity in hoxa10 or hoxa11 knockout mice.

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In patients affected by endometriosis the cyclic expression of HOXA10/HOXA11 and up-regulation during the window of implantation is missing and could partly explain the infertility related to the disease (Gui et al., 1999; Taylor et al., 1999b). Further studies evaluating the expression pattern of HOXA10/HOXA11 have suggested DNA methylation as a possible mechanism for altered gene expression. Three fragments were identified as methylated regions in the HOXA10 gene: the F1 region in the 5′ promoter region, and F2 and F3 that are located within the intron between exons 1 and 2 (Wu et al., 2005). Further investigation of these regions demonstrated that the F1 region showed a significant difference in methylation between baboons affected by endometriosis and disease-free animals, while the methylation status of the F2 and F3 regions did not reach statistical significance (Kim et al., 2007).

Hypermethylation of the 5′ promoter region of the hoxa10 gene and a down-regulation of HOXA10 and hoxa11 expression have been found in the eutopic endometrium of mice with induced endometriosis (Lee et al., 2009) and in humans with peritoneal endometriosis (Szczebańska et al., 2010) for the first time in luteal phase. This documented the hypermethylation of DNA as a mechanism responsible for silencing HOXA10 gene expression in women with mild peritoneal endometriosis. A recent study confirmed the HOXA10 hypermethylation in human eutopic endometrium in patients with endometriosis (Lu et al., 2013) and a recent study conducted by our research group found the HOXA10 gene hypermethylated in the mid-luteal endometrium from women with ovarian endometriomas (Fambrini et al., 2013).

However, to date, studies in humans comparing HOXA10 gene methylation in the endometrium from different ectopic sites in women affected by endometriosis are missing. Thus, the objective of the present study was to investigate whether the methylation status of the promoter region of the HOXA10 gene differs in eutopic and ectopic endometrium.

Materials and Methods

The study was conducted at the Department of Gynaecology, Perinatology and Human Reproduction, University Hospital, Florence, Italy between November 2010 and July 2013.

Tissue collection

Tissue from endometriotic ectopic lesions was collected during the laparoscopic session and the eutopic endometrium was obtained by endometrial biopsy in 18 women (aged 25–40 years) admitted for surgery with pain-related endometriosis (cases). For controls, endometrial biopsies were collected from 12 women (aged 26–41 years) undergoing laparoscopic surgery for other indications (6 for ovarian pathology of dermoid or simple ovarian cyst and 6 for subserous or intramural uterine myoma). The endometrial biopsies were obtained using an Endoram device (RI-MOS, Modena, Italy) or Novak curette. Histology was used to confirm the presence of ectopic endometriosis in the case group. All the 18 patients with endometriosis had Stage III–IV disease, as classified by the ASRM (American Society for Reproductive Medicine, 1997). For this pilot study a power calculation was not performed.

Women included in the study all had a normal cycle length (28–32 days) and had been without any hormonal treatment or the use of intrauterine contraception for at least 3 months. All samples were obtained in the luteal phase of the menstrual cycle (Days 17–25) and eutopic and ectopic endometrium were pared. No analysis of plasma hormone levels was performed. The endometrial biopsies underwent immediate processing for DNA extraction and were fresh frozen and stored at −80°C. Table I shows the disease distribution, age and fertility status of all women included in this study.

Sodium bisulfite DNA modification

Incubation of the target DNA with high bisulfite salt concentrations results in conversion of unmethylated cytosine residues into uracil, leaving the methylated cytosines unchanged. Therefore, bisulfite treatment gives rise to different DNA sequences for methylated and unmethylated DNA.

For methylation analysis, genomic DNA was extracted from the specimens of endometriotic ectopic tissue, the fragments of the eutopic endometrium from the same patients and the endometrial biopsies from controls, after an overnight digestion with proteinase K at 56°C, using an automated system (BioRobot EZ1, Qiagen, Germany).

For each sample 1 µg of genomic DNA was modified with sodium bisulfite conversion according to the manufacturer’s protocol (EpiTect Bisulfite Kit, Qiagen, Germany).

PCR amplification

For each patient, DNA samples were examined from the endometriotic ectopic tissue and from the eutopic endometrium.

DNA amplification of HOXA10 was carried out in a reaction volume of 50 µl, containing 2 µl of forward and reverse primers (10 µM) and 5 µl of bisulfite-treated DNA. As internal controls we used samples of methylated and non-methylated bisulfite-treated DNA (Qiagen), while nuclease-free water was used as a negative control.

Two primers that identified a CpG-rich fragment within the human HOXA10 gene promoter in the 5′ region upstream of exon 1 (the F1 region) were used, as described by Wu et al. (2005).
Amplification conditions were as follows: 95°C for 1 min followed by 40 cycles at 95°C for 30 s, 55°C for 30 s and 72°C for 30 s. A final extension at 72°C for 10 min was then performed. The 261 bp PCR products were checked by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining.

**Pyrosequencing methylation analysis**

The 261 bp region amplified was analyzed using Pyrosequencing technology (Biotage, Westborough, MA, USA). Two sequencing primers were designed using the Assay Design software 1.0 (Biotage): sequences 1 and 2, identifying 11 and 8 CpG sites, respectively:

Forward: 5′-TGGGGTAGTTTTTATAGTTTTTG-3′

Reverse: 5′-AACCCTTTCTAACTAACATTCTT-3′ (biotinylated).

Methylation was analyzed and quantified using the PyroMark ID pyrosequencing system and Pyro Gold reagents (Biotage). Methylation status is presented as a percentage of average methylation in all observed CpG sites.

**Statistical analysis**

Student’s t-tests were used to compare gene expression between the groups. Two-tailed P values < 0.05 were considered significant. SPSS was used as statistical software package.

**Ethical approval**

The study was approved by the ethics committee of Karolinska University Hospital as well as Careggi University Hospital. All participating women
gave written informed consent before being included in this study. The study has been conducted according to the principles of the Declaration of Helsinki.

## Results

Table I shows disease distribution and fertility status of the patients with endometriosis and controls.

Histologic analysis of endometrial samples revealed a normal secretory endometrium in all cases and controls. Endometriosis was confirmed in all ectopic samples and the tissue was of a sufficient amount for analyzing.

### DNA methylation of the HOXA10 gene

The methylation of the HOXA10 gene in eutopic and ectopic endometrium in patients is reported in the Supplementary data, Table SI. The mean methylation of sequences 1 and 2 in the eutopic endometrium was 5.1 and 5.9 (SD 2.1 and 2.3; SEM 0.5 and 0.5), respectively, and significantly higher when compared with the ectopic endometrium in women with endometriosis (sequence 1: 8.7 versus 5.1%; P = 0.001; sequence 2: 11.9 versus 5.9% P = 0.0001; mean 8.7 and 11.9 (SD 3.7 and 3.8; SEM 0.9 and 0.9, respectively, for sequences 1 and 2).

The eutopic endometrium of endometriosis patients was significantly more methylated when compared with the control group (sequence 1: 8.68 versus 6.25%; P = 0.037; sequence 2: 11.89 versus 9.25%; P = 0.032; (Fig. 1). No statistical difference was observed in the methylation level in cases with exclusively ovarian endometriosis when compared with cases with extraovarian involvement of the disease (Fig. 2).

Supplementary data, Figure S1 shows chromatograms reporting the methylation status of ectopic and eutopic endometrium in patients and control.

**Figure 1** Box plots showing methylation of sequences 1 and 2 (%) of the HOXA10 gene in eutopic and ectopic endometria in cases and control women.

**Figure 2** Box plots showing methylation of sequences 1 and 2 (%) of the HOXA10 gene in endometrioma and extraovarian endometriosis in women.

## Discussion

HOXA10 that encodes a transcriptional factor significant for successful implantation is expressed in endometrial glands and stroma throughout the menstrual cycle in humans. Previous studies have shown that HOXA10 gene expression is regulated by steroid hormones and shows a large increase in the mid-luteal phase, corresponding to the implantation window (Taylor et al., 1998, 1999a), an up-regulation that is lacking in patients affected by endometriosis (Gui et al., 1999; Taylor et al., 1999b). The protein expression of HOXA10 in endometriosis at locations outside of the normal HOXA10 expression domain has been studied (Browne and Taylor, 2006; Zanatta et al., 2010) but no study of methylation at these endometriosis locations has been previously carried out. Rackow and Taylor (2010) have recently found that the level of HOXA10 protein is decreased in the endometrium of patients with submucosal myomas, a finding that was in contrast to that in patients with intramural myomas.

Data on methylation of HOXA genes in humans are limited to one previous study, which demonstrated a hypermethylation of the promoter region of the HOXA10 gene in the eutopic endometrium that could be responsible for the aberrant expression of the gene in patients affected by endometriosis (Wu et al., 2005). No previous study has investigated the methylation profile of the HOXA10 gene in ectopic endometriotic tissue and whether the endometriotic lesions present a different methylation pattern in the eutopic endometrium. In this study we find that the eutopic endometrium in patients affected by endometriosis, in particular extraovarian endometriosis, is significantly more methylated compared with the control group. Although the fertility status of most patients in our study was unknown, this finding could explain, at least in part, the impaired fertility in patients affected by endometriosis.
HOXA10 protein expression has been demonstrated in peritoneal, previously not been addressed. To date in humans, altered ectopic endometrium and the character of the ectopic endometriotic disease has been demonstrated in human endometrium (Szczepani’ska et al., 2010) and protein levels were not investigated: it has been reported that laser capture micro-dissection could have provided an even better understanding of DNA methylation in these cell types.

In this study we have used whole biopsies. However, studying the expression of HOXA10 separately in the stromal and glandular compartment after laser capture micro-dissection could have provided an even better understanding of DNA methylation in these cell types.

Only the methylation status of the HOXA10 gene was addressed in this study and protein levels were not investigated: it has been reported that hypermethylation of the HOXA10 gene decreases the gene expression in human endometrium (Szczepani’ska et al., 2010).

Whether there is a correlation between the methylation status of the endometrium and the character of the ectopic endometriotic disease has previously not been addressed. To date in humans, altered ectopic HOXA10 protein expression has been demonstrated in peritoneal, ovarian and lung endometriosis (Browne and Taylor, 2006). A recent study found modified HOXA10 protein levels in the endometrium of women with superficial, ovarian and deep infiltrating endometriosis (Matsuzaki et al., 2009) but the methylation status was not studied.

In conclusion, this pilot study demonstrated a significantly altered methylation profile in eutopic versus ectopic endometrial tissue in humans. These findings could explain, in part, the compromised fertility in women affected by endometriosis. Demethylating agents to reverse aberrant methylation could represent an attractive field of future research to develop new epigenetic-based strategies for non-surgical treatment of infertility related to endometriosis.

Supplementary data
Supplementary data are available at http://humrep.oxfordjournals.org/.

Authors’ roles

Funding
No external funding was either sought or obtained for this study.

Conflict of interest
None declared.

References


