HOXA-10 expression in the mid-secretory endometrium of infertile patients with either endometriosis, uterine fibromas or unexplained infertility†

Sachiko Matsuzaki1,3, Michel Canis1, Claude Darcha2, Jean-Luc Pouly1, and Gérard Mage1

1CHU Clermont-Ferrand, Polyclinique-Hôtel-Dieu, Gynécologie Obstétrique et Médecine de la Reproduction, Boulevard Léon Malfreyt, 63058 Clermont-Ferrand, France 2CHU Clermont-Ferrand, Hôtel-Dieu, Service d’Anatomie et Cytologie Pathologiques, Clermont-Ferrand, France
3correspondence address. Fax: +33-4-73931706; E-mail: sachikoma@aol.com

BACKGROUND: The aim of this study was to investigate HOXA-10 expression in endometrium from infertile patients with different forms of endometriosis; with uterine fibromas, or with unexplained infertility and from normal fertile women.

METHODS: Expression levels of HOXA-10 mRNA and protein in endometrium were measured during the mid-secretory phase. This study utilized laser capture microdissection, real-time RT–PCR and immunohistochemistry.

RESULTS: HOXA-10 mRNA and protein expression levels in endometrial stromal cells were significantly lower in infertile patients with different types of endometriosis (deep infiltrating endometriosis, ovarian endometriosis and superficial peritoneal endometriosis), with uterine myoma, and unexplained infertility patients as compared with healthy fertile controls. HOXA-10 mRNA expression levels of micro-dissected glandular epithelial cells were significantly lower than those of microdissected stromal cells, without significant differences among the different groups. No protein expression was detected in glandular epithelial cells. The percentage of patients with altered protein expression of HOXA-10 in stromal cells were significantly higher in patients with only superficial peritoneal endometriosis (100%, 20/20, \( P < 0.05 \)) compared with the other infertile groups (deep infiltrating endometriosis: 72.7%, 16/22; ovarian endometriosis: 70.0%, 14/20; uterine myoma: 68.8%, 11/16; unexplained infertility: 55.6%, 5/9).

CONCLUSION: The present findings suggested that altered expression of HOXA-10 in endometrial stromal cells during the window of implantation may be one of the potential molecular mechanisms of infertility in infertile patients, particularly in patients with only superficial peritoneal endometriosis. One of the underlying causes of infertility in patients with only superficial endometriosis may be altered expression of HOXA-10 in endometrial stromal cells.

Key words: HOXA 10 / endometriosis / endometrium / female infertility

Introduction

Endometriosis is a common, benign, gynecologic disorder associated with pelvic pain and infertility. It is characterized by the presence of uterine endometrial tissue outside of the normal location. It affects approximately 10–15% of women of reproductive age and 25–50% of all women with infertility and 30–50% of women with endometriosis are infertile (Giudice and Kao, 2004). However, the underlying mechanisms of endometriosis-associated infertility remain unclear (Giudice and Kao, 2004). Many factors may be involved, and studies suggest that endometrial molecular defects involved in implantation during the implantation window might be one cause of endometriosis-associated infertility (Giudice et al, 2002; Giudice and Kao, 2004). However, the results of basic and clinical studies in
HOXA-10 expression in infertile patients

Materials and Methods

Patients

Patients undergoing laparoscopy for infertility were recruited at the Polyclinique de l’Hôpital Dieu, CHU Clermont-Ferrand, France. Inclusion criteria were: (i) regular menstrual cycles (between 26 and 32 days) with confirmation of menstrual history; (ii) age <38 years; (iii) minimum 2 years of infertility with current desire for conception, and (iv) no hormonal treatments such as gonadotrophin-releasing hormone agonists or sex steroids, and no use of intrauterine contraception for at least 6 months prior to surgery. Exclusion criteria were (i) bilateral tubal occlusion, (ii) patients with male factor infertility and (iii) mechanical distortion of the endometrial cavity by fibroids. Of these patients, we included those with endometriosis, UF, or UI in the present study. All included patients with endometriosis had surgical and histological diagnosis of endometriosis. We included infertile patients with DE, those with OE and those with SE. DE was defined as endometriosis deeper than 5 mm under the peritoneal surface. SE was defined as endometriosis located on the peritoneal surface. The OE group consisted of infertile patients with endometriotic ovarian cysts of more than 3 cm in diameter in the present study. The severity of the endometriosis was scored according to the revised American Society for Reproductive Medicine classification (rASRM) (American Society for Reproductive Medicine, 1997). According to the inclusion and exclusion criteria, infertile patients with submucosal myoma were excluded. Infertile patients who had no abnormal laboratory findings as well as no abnormal surgical findings were included in the UI group (Werbrouck et al., 2006). In addition, endometrial tissues were obtained from healthy fertile women with macroscopically normal pelvic cavities who underwent laparoscopic tubal ligation or reversal of tubal sterilization.

Published endometrial dating criteria (Noyes et al., 1950), menstrual history and serum progesterone levels were used to assess the menstrual cycle phase. Endometrial dating was performed independently by C.D. and an independent pathologist. All patients, independent of group, were selected for the present study on the basis of consistent histological findings, menstrual history and serum 17β estradiol and progesterone levels. Finally, endometrial samples (Days 20–23) from a total of 87 infertile patients and 20 healthy fertile women were included in the study. Of these, 22 patients had DE, 20 patients had OE, 20 patients had SE, 16 patients had UF (intramural) without endometriosis and nine patients were diagnosed with UI. Clinical characteristics of patients are shown in Table I. There were no significant differences in serum 17β estradiol and progesterone levels among different groups (Table I). The serum progesterone levels suggested that, in all patients, ovulation had occurred (DE: 21.8 ± 7.0, OE: 20.6 ± 5.1, SE: 20.8 ± 5.4, UF: 18.9 ± 6.4, UI: 22.8 ± 7.4, healthy fertile women: 19.6 ± 5.1 ng/ml, mean ± SD; Table I). Samples from 12 patients with DE, 10 with OE, 10 with SE, 10 with UF, 8 with UI and 12 healthy fertile women were analyzed by both real-time RT–PCR and immunohistochemical methods. The remaining samples were analyzed only by immunohistochemistry.

Endometrial tissue biopsies were performed just prior to surgery using an endometrial suction catheter (Pipelle, Laboratoire CCD, Paris, France). Each sample was divided into two portions. The first tissue portion was fixed in 10% formalin-acetic acid and embedded in paraffin for histopathological examination. The second portion was immediately collected in RNAlater (Ambion, Cambridge, UK) and stored at −20°C until further analysis was performed. All tissue samples were obtained with full and informed patient consent. The research protocol was approved by the Consultative Committee for Protection of Persons in Biomedical Research of the Auvergne (France) region.

Laser capture microdissection and RNA extraction

Laser capture microdissection (LCM) was performed as previously described (Matsuzaki et al., 2004, 2005). From each fresh frozen tissue sample, 10-μm thick frozen sections were prepared. The sections were mounted on positively charged slides (Super frost Plus, Menzel GmbH, Braunschweig, Germany). Sample preparation of frozen sections was performed using the National Cancer Institute protocol (http://rgap.nichd.nih.
Table I  Clinical characteristics of patients

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<th></th>
<th>Infertile patients</th>
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<th>Uterine fibroma</th>
<th>Unexplained infertility</th>
<th>Healthy fertile women</th>
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<td></td>
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<td>DE</td>
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<td>20</td>
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<td>Age*</td>
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<td>4.5 (2–8)</td>
<td>3 (2–4)</td>
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<td>rASRM stage*</td>
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<td>Posterior</td>
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<td>Type of dominant myoma</td>
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<td>Intramural</td>
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<td>Subserosal</td>
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<td>Serum progesterone levels (ng/ml)*</td>
<td>21.8 ± 7.0</td>
<td>20.6 ± 5.1</td>
<td>20.8 ± 5.4</td>
<td>18.8 ± 6.4</td>
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<td>Serum 17ß estradiol levels (pg/ml)*</td>
<td>107.7 ± 36.2</td>
<td>109.4 ± 33.7</td>
<td>112.7 ± 36.8</td>
<td>113.5 ± 33.1</td>
<td>103.6 ± 30.8</td>
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| DE: patients with deep infiltrating endometriosis without ovarian endometriosis; OE: patients with ovarian endometriosis without deep infiltrating endometriosis; SE: patients with only superficial peritoneal endometriosis.  
*Median (range).  
**Revised American Society for Reproductive Medicine classification (rASRM) (American Society for Reproductive Medicine, 1997).  
†Mean ± SD.
HOXA-10 expression in infertile patients

Quantitative real-time RT–PCR

Quantitative real-time RT–PCR with a Light Cycler was performed on total RNA from microdissected tissues as previously described (Matsuzaki et al., 2005, 2006). Total RNA (50 ng) was subjected to an RT reaction using Superscript II Reverse Transcriptase (Invitrogen). Quantitative real-time PCR was performed in a Light Cycler System using the FastStart DNA Master SYBR Green I kit as recommended by the manufacturer (Roche, Mannheim, Germany). In a total volume of 20 μl, each reaction contained 2 μl SYBR green I reaction mix (containing of Taq DNA-polymerase reaction buffer, dNTP mix, SYBR green I, MgCl2, and Taq DNA polymerase), 0.5 μM of primer (HOXA-10: forward 5′-CTG AGG TCA ATG GTG CAA AGG A-3′, reverse 5′-TTT GCC AAC CTG CAT GTC CA-3′; GAPDH: forward 5′-TCGACCACCAA CTGCTTAG-3′, reverse 5′-CAGCCAGGAGGATGATGTC-3′), 4 mM MgCl2 and 2 μl cDNA, and standard or nuclease free water as a negative control. Primers were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA). Quantification of the targets in the unknown samples was performed using a relative quantification method with external standards. The target concentration was expressed relative to the concentration of a reference housekeeping gene, GAPDH. After each run, a melting curve analysis was performed to verify the specificity of the PCR reaction. The size of the expected RT–PCR product (161 bp) was confirmed by ethidium bromide-stained agarose gel electrophoresis. The procedure was repeated three times independently to ensure the reproducibility of the results. All of the samples with a cycle threshold coefficient of variation value higher than 5% were retested.

Immunohistochemistry

Using the following protocol, immunohistochemical staining was performed on paraffin sections with goat polyclonal antibody directed against HOXA-10 (sc-17159, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Sections were deparaffinized and antigen retrieval was performed in 0.01 M sodium citrate (pH 6.0) for 3 min at full pressure using a pressure cooker. Sections were then rinsed in distilled water and treated with 3% hydrogen peroxide solution for 5 min to inhibit endogenous peroxidase activity. After rinsing in 0.01 M phosphate-buffered saline (PBS, pH 7.2), sections were incubated overnight at 4°C with the primary antibody (diluted 1:400 with 3% bovine serum albumin). Term decidual tissues were included in each staining run as positive controls (Sarno et al., 2006). Negative controls were performed by replacing primary antibodies with normal goat IgG diluted to the same concentration. After rinsing in PBS, sections were incubated with biotinylated anti-rabbit, anti-mouse and anti-goat immunoglobulins (DAKO LSAB+ System, DAKO Corp.) for 15 min. After washing in PBS, sections were incubated with streptavidin conjugated to horseradish peroxidase (DAKO LSAB+ System) for 15 min. Sections were then washed with PBS, colored with aminolaevulinate substrate, counterstained with Mayer’s hematoxylin, and mounted.

Quantification of HOX 10 immunostained cells

To quantify immunostained cells objectively, we applied a computerized image analysis system which consisted of a light microscope (Leica, Lyon, France) (X 40 objective, X 10 ocular) with a color charge coupling device camera (Sony, Paris, France) connected to a Samba 2005 computer analysis system (Alcatel-TITN, Grenoble, France). The percentage of immunostained nuclear surface relative to the total nuclear surface for HOXA-10 was obtained as previously described (Matsuzaki et al., 2006). For all samples of endometrial tissue, 10 non-overlapping fields were analyzed.

Results

Quantitative real-time RT–PCR for HOXA-10 mRNA

HOXA-10 mRNA expression levels in endometrial glandular epithelial cells were significantly lower than those in stromal cells in each of the five groups (DE: P < 0.03, OE: P < 0.05, UF: P < 0.002, UI: P < 0.03, healthy fertile women: P < 0.0003, Mann–Whitney U-test). We detected no significant differences in endometrial glandular epithelial cell expression levels between the different groups (Fig. 1A, one-way ANOVA). Expression levels in endometrial stromal cells were significantly higher in the healthy fertile control group compared with those of the five infertile groups (Fig. 1B, one-way ANOVA).

Percentage of immunostained nuclear surface for HOXA-10

We detected immunolocalization of HOXA10 protein in endometrial stromal cells, whereas no immunolocalization was detected in endometrial glandular epithelial cells (Fig. 2). The percentage of immunostained nuclear surface (PI) in endometrial stromal cells was significantly higher in healthy fertile controls compared with that in any of the five infertile groups (Fig. 3, one-way ANOVA).
Superficial peritoneal endometriosis (100%, 20/20) compared with metrial stromal cells was significantly higher in patients with only percentage of patients with altered HOXA 10 expression of endometrial cells. Considering the minimum PI in stromal cells from a healthy fertile control (32.9%, Fig. 3), a percentage of patients with a PI lower in infertile patients with endometriosis, in those with myoma uteri and in UI patients compared with that in healthy fertile controls. In addition, the percentage of patients in the studied population with altered HOXA-10 protein expression was significantly higher in patients with only superficial peritoneal endometriosis compared with that of infertile patients with other types of endometriosis, those with UF, and UI patients. Tubal adhesions in patients with severe endometriosis can clearly cause infertility. However, the reason for infertility is less obvious in patients with only superficial peritoneal endometriosis that does not mechanically compromise tubal patency or function. The present findings as well as those by Lessey et al. (1994) suggested that one of the major underlying causes of infertility in patients with only superficial endometriosis might be implantation failure. However, HOXA-10 is not the sole molecule responsible for successful implantation (Giudice and Kao, 2004). Many different molecules could be involved in implantation failure and different underlying molecular defects might be involved in the molecular mechanisms of endometriosis-associated infertility (Giudice and Kao, 2004). Further studies to investigate other endometrial molecular defects during the window of implantation in infertile patients with endometriosis, particularly those who do not have altered expression levels of HOXA-10 protein, might shed light on the underlying mechanisms of endometriosis-associated infertility.

To date, it remains unknown why some patients with endometriosis are fertile, whereas others are not. Endometriosis is detected in 4–8% of cases during laparoscopic tubal ligation in women of proven fertility (D’Hooghe et al., 2003). If endometrial molecular defects are a consequence of the disease in women with endometriosis as demonstrated by baboon studies (Gashaw et al., 2006; Kim et al., 2007), the biological effect of each endometriotic lesion on eutopic endometrium might be different, and consequently only some women with endometriosis might have endometrial molecular defects which alter embryo implantation. Further studies are needed, both in vivo and in vitro, to investigate interactions between endometriotic tissues and eutopic endometrium and to confirm the baboon studies.

One mechanism by which HOXA10 levels are decreased in endometriosis is by the methylation of this gene. Wu et al. (2005) demonstrated that methylation of HOXA10 in the endometrium of women with stage III and IV endometriosis is aberrant compared with those without endometriosis. Baboon and mouse studies have demonstrated that the presence of peritoneal endometriotic lesions could induce aberrant methylation of HOXA10 in the eutopic endometrium (Kim et al., 2007; Lee et al., 2009). These studies have suggested that superficial peritoneal endometriotic lesions might induce aberrant methylation of the HOXA10 in eutopic endometrium, resulting in decreased HOXA10 protein expression in endometrial stromal cells.

**Discussion**

The present study demonstrated that both HOXA10 mRNA and protein expression levels in endometrial stromal cells were significantly lower in infertile patients with endometriosis, in those with myoma uteri and in UI patients compared with that in healthy fertile controls. In addition, the percentage of patients in the studied population with altered HOXA-10 protein expression was significantly higher in patients with only superficial peritoneal endometriosis compared with that of infertile patients with other types of endometriosis, those with UF, and UI patients. Tubal adhesions in patients with severe endometriosis can clearly cause infertility. However, the reason for infertility is less obvious in patients with only superficial peritoneal endometriosis that does not mechanically compromise tubal patency or function. The present findings as well as those by Lessey et al. (1994) suggested that one of the major underlying causes of infertility in patients with only superficial endometriosis might be implantation failure. However, HOXA-10 is not the sole molecule responsible for successful implantation (Giudice and Kao, 2004). Many different molecules could be involved in implantation failure and different underlying molecular defects might be involved in the molecular mechanisms of endometriosis-associated infertility (Giudice and Kao, 2004). Further studies to investigate other endometrial molecular defects during the window of implantation in infertile patients with endometriosis, particularly those who do not have altered expression levels of HOXA-10 protein, might shed light on the underlying mechanisms of endometriosis-associated infertility.

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Further studies are needed to investigate the effect of each endometriotic lesion, especially superficial peritoneal endometriosis, on methylation patterns of the HOXA10 gene in eutopic endometrium. HOXA-10 expression is also altered during the window of implantation in patients with hydrosalpinx (Cermik et al., 2003; Daftary et al., 2007) and PCOS. These findings and the present study suggest that altered expression of the HOXA-10 gene in the endometrium during the mid-secretory phase might be a common factor among patients with infertility due to different etiologies. Thus, the question of whether methylation of HOXA-10 gene is a common event in the endometrium of infertile patients might be raised. Further studies to investigate the possible mechanisms that could potentially alter the expression of HOXA-10 could shed light on the molecular mechanisms of implantation failure among infertile patients with different etiologies.

In the present study, we detected a significant difference in stromal HOXA-10 expression of mRNA and protein between unexplained infertile patients and normal fertile controls. However, we detected that some UI patients had normal expression levels, and consequently, the percentage of UI patients with altered HOXA-10 protein expression was significantly lower than that of patients with only superficial peritoneal endometriosis. Studies demonstrated that the UI group was more likely to achieve pregnancy after controlled ovarian hyperstimulation and intrauterine insemination (COH/IUI) (Omland et al., 1998) or after COH only (Nuojua-Huttunen et al., 1999) as compared with the minimal endometriosis-associated and tubal factor infertility groups. The endometriosis-associated infertility group consisted of untreated patients and patients who had received medical or surgical treatment for endometriosis (Omland et al., 1998; Nuojua-Huttunen et al., 1999). Although further studies are necessary to investigate endometrial molecular defects in UI patients, the present findings suggest that not all UI patients have endometrial
molecular defects, and thus, implantation in UI patients is better than that of patients with minimal endometriosis. On the other hand, Werbrouck et al. (2006) demonstrated no significant difference between UI patients and surgically treated minimal to mild endometriosis after COH/IUI treatment. In the study by Werbrouck et al. (2006), the endometriosis-associated infertility group consisted of surgically treated minimal to mild endometriosis. These findings might suggest that surgical treatment of superficial endometriosis lesions could improve “receptivity”. To date, it remains to be clarified whether surgical treatment of endometriosis improves reproductive outcome.

Dafary et al. (2007) demonstrated that endometrial HOXA-10 expression was down-regulated in infertile patients with hydrosalpinx compared with fertile women. They also demonstrated that endometrial HOXA-10 expression was up-regulated to normal levels 4 months after salpingectomy. Further studies are required to investigate whether surgical removal of endometriotic tissues, particularly in patients with only superficial peritoneal endometriosis, could modulate HOXA10 expression levels as well as other molecules involved in embryo implantation.

In the present study, we detected significantly lower expression levels of HOXA-10 in patients with uterine leiomyoma. A recent study demonstrated that HOXA-10 expression levels of mRNA and protein in patients with submucosal myomas are altered, whereas those of patients with intramural myomas are comparable to controls (Rackow and Taylor, 2008). However, they investigated HOXA-10 expression levels during the proliferative phase. As HOXA-10 expression in normal cycling endometrium is dramatically up-regulated during the mid-secretory phase compared with that of the proliferative phase (Taylor et al., 1998), it is therefore not clear if HOXA-10 expression during the mid-secretory phase is not altered in patients with intramural myomas. A recent microarray study provides evidence that intramural leiomyomas not affecting the endometrial cavity still alter the expression pattern of some endometrial genes, but the genes involved in implantation are not affected (Horcajadas et al., 2008). In the present study, HOXA-10 protein expression levels were significantly lower in infertile patients with UF compared with healthy fertile women. However, we detected 68.8% of patients with altered HOXA-10 expression, suggesting that only certain patients with UF might have alteration of HOXA-10 expression. The present findings and those by Horcajadas et al. (2008) suggest that not all patients with UF have endometrial molecular defects involved in implantation, but some patients do during the window of implantation. Considering the variety of endometrial molecules involved in implantation during the window of implantation (Dey et al., 2004), the population of infertile patients with UF might be heterogeneous. Before concluding that those patients with intramural leiomyomas not affecting the endometrial cavity do have endometrial molecular defects involved in implantation, a larger number of endometrial samples from patients with UF should be investigated.

In conclusion, the present study demonstrated that HOXA-10 mRNA and protein expression levels in endometrial stromal cells were significantly lower in infertile patients with different forms of endometriosis, patients with myoma uteri, and UI patients compared with healthy fertile control during the mid-secretory phase. In addition, the percentage of patients with altered HOXA-10 protein expression was significantly higher in infertile patients with only superficial peritoneal endometriosis compared with the other infertile groups in the present study population. Further studies are necessary to confirm the present findings in larger samples and to investigate whether other endometrial molecular defects involved in embryo implantation are also altered in each infertile group.

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