Altered expression of 3΄paralogus HOX A-D clusters in endometriosis disease: A case-control study

Masoumeh Golestan Jahromi1, 2 Ph.D., Reza Aflatoonian1 M.D., Ph.D., Parvaneh Afsharian2 Ph.D., Samaneh Aghajanpour1 M.Sc., Maryam Shahhoseini1 Ph.D., Abbas Aflatoonian1 M.D.

Introduction

Endometriosis is a gynecological disease, demonstrate by the presence of endometrial glands and stroma located in ectopic sites such as pelvic, peritoneum, ovaries, and rectovaginal septum. The most recognizable signs of the disease is pain such as chronic dysmenorrhea, intermenstrual abdominal and pelvic pain and back pain (1). The prevalence of endometriosis in the population is 6-10%; but in women with pain, infertility or both, increases to 35-60% (2). Endometriosis is considered as a complicated disorder and despite its high frequency; the etiology of the disease is not well understood. Moreover, the diagnostic and therapeutic approaches are not definitely known and more investigations are needed to identify its etiology (3).

Recent studies have suggested that abnormalities in the regulation of specific genes are involved in the incidence of endometriosis (4-7). In women with particular genetic backgrounds, specific networks of genes are involved in endometrial tissue formation (8). During mammalian evolution, specification of various parts of body along the axis, from the branchial area through to the tail, is controlled by HOX genes (9).
In vertebrates, the 39 HOX genes are classified into four clusters according to their location on the chromosomes: HOXA, HOXB, HOXC and HOXD (10). Scientists subdivided HOX genes within the HOXA-D clusters into 13 paralogous groups. Genes in each paralogous group have functional similarity with corresponding paralogous genes in other clusters (11). Duboule and colleagues determined that the expression pattern of HOX genes are sequentially from 3’ to 5’ along the anterior-posterior (AP) axis, during embryonic development, and the 3’ genes are expressed prior to the 5’ genes (12).

Each adult organ displays a specific grouping of HOX genes expression representing the molecular portrait (projection) of the organ (13). Furthermore, specific sets of homeobox genes regulate reproductive function in the adult (14), for example they have an important function in the regulation of cyclic endometrial regeneration (15). Furthermore, HOXA10 and HOXA11 regulate endometrial receptivity and also HOXC and HOXD genes have a role in the early development of endometrium and endometrial proliferation. Therefore, the network of HOX genes may be involved in multiple aspects of endometrial development and function, such as proliferation and differentiation (16). Due to this background, it is supposed that HOX genes have a functional role in endometriosis development.

The aim of this study was identifying the expression pattern of 1-5 paralogous HOX genes in endometriosis (both eutopic and ectopic tissues) and comparing it to the endometrium of healthy women.

Materials and methods

Participants and tissue collection

This case-control study was performed at the Research and Clinical Center for Infertility, Yazd, Iran and Royan Institute, Tehran, Iran. Fifteen women scheduled for surgery of endometriosis participated in this study. To minimize the genetic heterogeneity, one sample of endometriomas and one sample of eutopic endometrium have been collected from each participant in the proliferation phase. Also, fifteen healthy women were enrolled in this study as the control group. These women who confirmed to have no other causes of infertility were laparoscopically checked for the absence of endometriosis.

Women participated in this study had normal menstruation cycle, not receiving hormone therapy from 3 months before the surgery and were younger than 37 yr. All patients were in the follicular phase of the menstrual cycle at the time of the surgical procedure. Samples of endometriosis tissue were obtained from ectopic sites in the abdomen; all samples used in this study were endometriomas. In each patient, the cyst wall of the endometrioma was removed during laparoscopy. Eutopic endometrial tissues were obtained from the uterus of the same patients at the time of the laparoscopy by performing hysteroscopy, followed by dilatation and curettage. Samples of the control group were attained during the hysteroscopy by curettage.

All of the samples were immediately placed in RNA protection reagent, RNAlater (Ambion, Austin, TX), frozen in liquid nitrogen and stored at -80°C.

RNA extraction and cDNA synthesis

QIAGEN RNAeasy kit (QIAGEN, Venlo, the Netherlands) was used to extract mRNA according to the manufacturer’s instructions. RNA quality and concentration were measured using Nanodrop 2000 (Thermo, USA). cDNA synthesis was performed with the Super Script double-stranded cDNA synthesis kit (QIAGEN, Venlo, The Netherlands). Two thousand nanograms of total RNA were used for cDNA synthesis according to the manufacturer’s instruction of cDNA synthesis kit (Qiagen, Cat.No:207045). RT-PCR was performed with twenty-five nanogram of cDNA and human-specific primers. An oligonucleotide primer for specified

amplification of each candidate gene was designed and tested. The sequences and annealing temperatures for each primer are listed in Table I.

**Real-time polymerase chain reaction**

Quantitative polymerase chain reaction (q-PCR) was performed on the prepared cDNA samples with the use of primers designed for HOXA1, HOXA2, HOXA3, HOXA4, HOXA5, HOXB1, HOXB2, HOXB3, HOXB4, HOXB5, HOXC4, HOXC5, HOXD1, HOXD3, and HOXD4. A melting curve was generated after each run to verify the specificity of the primers, shown by the presence of a single band and no primer-dimer artifacts. Each reaction of the PCR plate contained 10 µL SYBR green (PCR Master Mix, 5 µL; Applied Biosystems), 6 µL molecular water, 1 µL of each primer (20 pmol), and 2 µL cDNA (final concentration equal to 25 nanograms per µL). Real-time PCR was performed under standard conditions, and all experiments were run in triplicate. The quantitative PCR data were analyzed with the use of the comparative cycle time (CT) method (17). The difference in cycle times, ΔCT, was determined as the difference between the tested gene and the housekeeping gene (β-actin). Then the ΔΔCT was obtained by finding the difference between patients and control groups.

Reactions with a threshold cycle >35 were considered to be undetectable. Fold-change ratios between groups were derived and the 2-fold difference was applied to select up-regulated (fold ≥2) and down-regulated (fold ≤2) genes. The relative quantity of gene of interest was normalized to the relative quantity of β-actin, as reference gene and was reported as fold change of gene expression.

**Ethical consideration**

The study protocol was approved in both Yazd Research and Clinical Center for Infertility and ROYAN Institute ethics committees (EC/91/1131). Informed consent was obtained from all participants for the use of their tissue samples.

**Statistical analysis**

The values were expressed as mean±SEM. The data were analyzed by one-way ANOVA using SPSS (Statistical Package for the Social Sciences, version 21.0, SPSS Inc, Chicago, Illinois, USA) software, followed by Tukey’s test analysis to compare various groups with each other. The statistical significant difference was defined as p <0.05.

**Results**

Expression of 3’paralogus genes in HOXA (1, 2, 3, 4, and 5), HOXB (1, 2, 3, 4, and 5), HOXC (4, and 5), and HOXD (1, 3, and 4) clusters were quantitatively evaluated in normal endometrium of healthy women, compare to eutopic and ectopic tissue of endometriosis patients.

In the way, HOXA3, HOXA4, and HOXA5 showed significant down-regulation in ectopic group compare to the control group (p=0.0002, 0.0049, and 0.0032 respectively), also there was significant down-regulation in eutopic group compare to the control group (p=0.009, 0.0052, and 0.0033), HOXA2 was significantly down-regulated in ectopic group compare to the eutopic and control group (p=0.0045, and 0.0050 respectively), however, there was no significant difference in mRNA expression of HOXA1 between all groups.

HOXA cluster genes showed an overall down-regulation pattern in eutopic and ectopic groups compare to the control group (Figure 1); similarly, genes in the HOXB cluster showed significant down-regulation in eutopic and ectopic groups compare to the control group (Figure 2).

HOXB1, 2, 3, 4, and 5 was significantly down-regulation in ectopic group compare to the control group (p=0.003, 0.0021, 0.004, 0.0006, and 0.0002 respectively). Two HOX genes in B cluster (HOXB4 and HOXB5) showed significantly down-regulation in eutopic groups compare to the control group (p=0.0007 and p=0.0002 respectively). Also, our data showed a significant decrease in the expression level of HOXB3, HOXB4, and
HOXB5 in ectopic tissues compare to the eutopic endometrium (p=0.0045, 0.0083, and 0.0076 respectively). Interestingly, the expression level of all HOX paralogous in this cluster was significantly lower in ectopic tissue compare to the eutopic endometrium.

In HOXC cluster, 3’ paralogous genes showed overall significant up-regulation in eutopic and ectopic group compared to the control group. There was a significant increase in mRNA expression level of HOXC4 and HOXC5 in ectopic tissues compare to the eutopic endometrium (p=0.0005 and p=0.006 respectively) (Figure 3). HOXC4 and HOXC5 were significantly over-expressed in ectopic compared to control group (p=0.0002 and 0.0005 respectively), also, there was a significant increase in mRNA expression level of HOXC4 and HOXC5 in eutopic group compare to the control group (p=0.019 and 0.0033 respectively). HOXD1 and HOXD3 genes also showed significant up-regulation in eutopic and ectopic group compare to the control group (p<0.05, Figure 3). HOXD4 was significantly over-expressed in ectopic compare to control group, but there were no significant changes in its mRNA level in the eutopic tissues compared to the control group.

Table I. Sequence of the primers used for qPCR experiments

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (F)</th>
<th>Reverse primer (R)</th>
<th>Product size (bp)</th>
<th>Access number</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>F: 5’ CAAGATCATCTGCTCTCTCCTG 3’</td>
<td>R: 5’ ATCCACATCTGCTGAAGG 3’</td>
<td>151</td>
<td>NM_001101.4</td>
</tr>
<tr>
<td>HOXA1</td>
<td>F: 5’ ACCCAACAAGAAGCCT 3’</td>
<td>R: 5’ TACTCTCACAATTCCTCCTG 3’</td>
<td>113</td>
<td>NM_153620.2</td>
</tr>
<tr>
<td>HOXA2</td>
<td>F: 5’ AGAGGACGAGGAGAAGAG 3’</td>
<td>R: 5’ ACTGGAAGACCTTGGAGGAG 3’</td>
<td>151</td>
<td>NM_006735.3</td>
</tr>
<tr>
<td>HOXA3</td>
<td>F: 5’ TTCCCTGCTCTCCTCCTC 3’</td>
<td>R: 5’ CCATTCAGGACCAAAAGAG 3’</td>
<td>125</td>
<td>NM_153631.2</td>
</tr>
<tr>
<td>HOXA4</td>
<td>F: 5’ AGATGAAACTGGAAGAAGG 3’</td>
<td>R: 5’ GGGATGGAAGAGGTGAGG 3’</td>
<td>121</td>
<td>NM_002144.1</td>
</tr>
<tr>
<td>HOXA5</td>
<td>F: 5’ GAGCCGAAACTCAGACAC 3’</td>
<td>R: 5’ CGCCGAGTCCCTGAAAT 3’</td>
<td>148</td>
<td>NM_019102.3</td>
</tr>
<tr>
<td>HOXB1</td>
<td>F: 5’ AAACCCCAACCGACGAGACG 3’</td>
<td>R: 5’ GCAATCTCCACCTCCCTC 3’</td>
<td>150</td>
<td>NM_002144.3</td>
</tr>
<tr>
<td>HOXB2</td>
<td>F: 5’ GCCACGTCCTCCTCCTC 3’</td>
<td>R: 5’ CTTCATCAGGTCAGAGCAG 3’</td>
<td>150</td>
<td>NM_002145.3</td>
</tr>
<tr>
<td>HOXB3</td>
<td>F: 5’ TTTTTCATCTCCTCCTCCTC 3’</td>
<td>R: 5’ CACATCCTGCTGAGCCC 3’</td>
<td>151</td>
<td>NM_00133023.1</td>
</tr>
<tr>
<td>HOXB4</td>
<td>F: 5’ AGACAGAAAAGAATAAGAGGAG 3’</td>
<td>R: 5’ CGGCCAGAGGAAACCAAGAC 3’</td>
<td>120</td>
<td>NM_024015.4</td>
</tr>
<tr>
<td>HOXB5</td>
<td>F: 5’ TATACCCCGTCTACAGACC 3’</td>
<td>R: 5’ GGTGCTCTTCTCCTTACTCC 3’</td>
<td>159</td>
<td>NM_002147.3</td>
</tr>
<tr>
<td>HOXC4</td>
<td>F: 5’ TCTCTTCTCCTCCACTC 3’</td>
<td>R: 5’ CCCGAGCACTACACCTTG 3’</td>
<td>159</td>
<td>NM_014620.5</td>
</tr>
<tr>
<td>HOXC5</td>
<td>F: 5’ TCCATAGTGCCATCCACTTACC 3’</td>
<td>R: 5’ TCCATAGTGCCATCCACTTACC 3’</td>
<td>148</td>
<td>NM_018953.3</td>
</tr>
<tr>
<td>HOXD1</td>
<td>F: 5’ GGTGCTCTCCTCCTCCTC 3’</td>
<td>R: 5’ GCCATCCAGTCCACCTCCT 3’</td>
<td>114</td>
<td>NM_024501.2</td>
</tr>
<tr>
<td>HOXD3</td>
<td>F: 5’ AAAGAAACCTGGAAGAGCCTC 3’</td>
<td>R: 5’ CACGTCCACCCTCAGTGG 3’</td>
<td>141</td>
<td>NM_006898.4</td>
</tr>
<tr>
<td>HOXD4</td>
<td>F: 5’ CAGGAGATGAGAAGTGGA 3’</td>
<td>R: 5’ CGTGTGCGGGTGTCTTTGGG 3’</td>
<td>138</td>
<td>NM_014621.2</td>
</tr>
</tbody>
</table>
Figure 1. Relative gene expression of HOXA cluster genes in ectopic and eutopic tissues of endometriosis patients (n=15) compared to healthy women (n=15). The relative quantity of gene of interest was normalized to the relative quantity of β-actin, as reference gene and was reported as fold change of gene expression. Values expressed as mean ± SEM. Data were analyzed by ANOVA followed by Tukey’s multiple comparison test. (*p<0.05 compared to control group, ** p<0.05 between eutopic and ectopic groups).

Figure 2. Relative gene expression of HOXB cluster genes in ectopic and eutopic tissues of endometriosis patients (n=15) compared to healthy women (n=15). The relative quantity of gene of interest was normalized to the relative quantity of β-actin, as reference gene and was reported as fold change of gene expression. Values expressed as mean±SEM. Data were analyzed by ANOVA followed by Tukey’s multiple comparison tests. (*p<0.05 compared to control group, ** p<0.05 between eutopic and ectopic groups).
Discussion

Endometriosis still remains a significantly under-diagnosed and under-treated disease (18). There are numerous individual and public health concerns about the endometriosis and its treatment. Therefore, it is very important to understand its pathogenesis to help the prevention of the disease and also development of new and effective treatment strategies (19). The current study was undertaken to identify differentially expressed 3′ HOX genes paralogous in all four clusters in endometriosis compare to normal endometrium. An important finding of this study is a systematic alteration pattern of HOX genes clusters in endometriosis based on their cluster (down-regulation of HOXA, HOXB and up-regulation of HOXC and HOXD) in ectopic and eutopic tissue. Interestingly, during the current study we detected the meaningful difference levels in the expression of 3′ HOX gene members in the endometrium of the study groups. As there were more than 150-fold change in HOXC cluster besides more than 400-fold change in HOXB cluster. While we expected a very low expression level of these paralogous HOX genes in uterus and endometriosis implants due to their loci on chromosome. In addition, these genes specify and limited to the head and neck (20, 21) and naturally have not an important role in uterus development or etiology of related disease. HOX genes within the different clusters are classified as belonging to the one of 13 paralogous groups (Hox1-Hox13), based on their sequence similarities and relative positions in the loci, and a single cluster contains only a subset of the 13 groups. The spatial and temporal expression of HOX genes along the anterior-posterior axis during embryonic development is highly related to their physical organization (22, 23). Numerous studies investigated the role of HOX genes in endometriosis (15, 24, 25). Most of them explored the paralogous of 9-13 in each cluster (26, 27). According to the cohort of studies, there is no evidence for definite expression and function of all 3′HOX genes in 4 clusters in the limb and pelvic organs such as the uterus. The majority of these HOX genes paralogous are expressed in the central nervous system (CNS), where they have critical functions in neuronal specification and target connectivity (20, 21). However during the current study, we detected the expression of 3′ HOX genes in the endometrium, with high levels of changes in expression of these members between study groups. These data hypothesized the functional role of 3′ paralogous HOX genes in uterus organogenesis and endometriosis pathology. Finally, according to our data, the altered expression of specific HOX genes appeared in ectopic and eutopic endometrial tissues compared to the normal endometrium.
These mutual features between eutopic endometrium and ectopic tissue which are not detected in the endometrium of women without endometriosis may approve the hypothesis that the main defect inducing endometriosis such as alteration of gene expression could be occur in the eutopic endometrium or in the uterus.

**Conclusion**

In summary, despite recent progress in the investigation of the different features of endometriosis, it still remains a debilitating disorder that affecting a large cohort of women. Our study showed that 1-5 paralogous HOX genes have different expression pattern in endometriotic tissue compare to eutopic and control tissues. Therefore our result revealed some unknown aspect of genes expression aberration in endometriosis. It is to be expected that new therapies will be established based on the molecular targets summarized above.

**Acknowledgments**

The authors would like to thank all the patients that consented to participate in this study as well as the staff of the operation room of Madar private Hospital in Yazd for all their help. This article was financially supported by Research and Clinical Center for Infertility, Yazd, Iran and Royan Institute, Tehran, Iran (No: 91000408).

**Conflict of interest**

There is no conflict of interest in this study.

**References**


