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Evaluation of Gene Expression of *ISL2, VAX1, BARX1,* and *OTX2* in Follicular Fluid of Endometriosis Patients Undergoing IVF/ICSI Treatment

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ABSTRACT:
Background and Objective: Endometriosis is a chronic, pre-inflammatory disease characterized by the growth of tissue resembling the endometrium outside the uterus, affecting approximately 10% of women worldwide. *HOX* family genes and their cofactors play a crucial role in the growth and maturation of the female reproductive system. This study aims to evaluate the expression of *ISL2, VAX1, BARX1,* and *OTX2* genes in the follicular fluid of endometriosis patients undergoing IVF/ICSI cycles. **Materials and Methods:** Follicular fluid samples were collected from 20 women with endometriosis and 20 healthy women (non-endometriosis) who visited Royan Institute for infertility treatment. After reviewing inclusion and exclusion criteria and obtaining written consent, VAX1, ISL2, BARX1, and OTX2 gene expression levels were assessed and compared between the two groups using Real-time PCR. **Results:** The results from Real-time PCR indicated that the expression levels of *OTX2* and *ISL2* were significantly increased ($p<0.05$). At the same time, *BARX1* and *VAX1* showed a significant increase ($p<0.01$) in the follicular fluid of endometriosis patients compared to healthy women. **Conclusion:** The findings suggest that the expression levels of *VAX1, ISL2, BARX1,* and *OTX2* are elevated in the follicular fluid of patients with endometriosis.

Keywords: Endometriosis, follicular fluid, HOX gene cofactors, gene expression, IVF/ICSI.

INTRODUCTION:

Endometriosis is a multifactorial disease in which many genetic, hormonal, and environmental factors play a role in its development. This disease is associated with the growth of tissue lesions similar to endometrium in different places outside the uterus. It affects about 10% of women of reproductive age, which can be detected in 40-50% of women with pelvic pain or infertility(1). Menstrual disorders, nausea, dizziness and headache, anxiety, fatigue, and depression are symptoms of endometriosis(2). Laparoscopy, MRI, and CT scan are among the methods of diagnosing this disease, and in recent years, ultrasound has replaced the laparoscopic procedure and is used to diagnose this disease. These methods are invasive. So far, much research has been done to identify biomarkers so that non-invasive techniques can be used to diagnose endometriosis(3). In women's reproductive system's growth and maturation, all types of *HOX* family genes and their cofactors play an important role(4).

To investigate the process and mechanism of endometriosis, the methylation changes of *HOX* family genes in this disease were examined and homeobox genes, which are the main growth control genes and play a role in cell differentiation, were identified as effective in the development of endometriosis(5, 6). According to the most accepted hypothesis about the origin of endometriosis (i.e. retrograde menstruation), the stromal and epithelial cells from the endometrial tissue shed by retrograde flow during menstruation reach the ovaries or peritoneum. They can cause endometriosis lesions, The growing and developing endometriosis lesion causes angiogenesis and neurogenesis, at this time it secretes chemical absorbent molecules during which several macrophages and NK cells are absorbed by reducing their activity. Then, methylation changes in DNA

increase the expression of estrogen receptor beta and decrease the expression of progesterone receptor, which will cause the growth and maintenance of endometriosis lesions. On the other hand, increasing the expression of the beta estrogen receptor leads to the induction of the expression of genes that encode inflammatory molecules, including the COX2 molecule(7).

HOX cluster genes play an important role in the development of tissues and reproductive organs(4). *BARX, VAX1, ISL2,* and *OTX2* genes are among the cofactor genes of the *HOX* family. These genes all encode proteins and are expressed more than 2000 times in ectopic tissues and are involved in reproduction and diseases such as reproductive cancers and breast cancer, etc. have a role(7). In the study of Esfandiari et al. in 2021, the methylation and expression of these four categories of the *HOX* gene family and the cofactors of these genes were investigated in the tissue and organoids of ectopic and eutopic endometriosis compared to healthy endometrium. Based on this study, the branches of *HOX* genes and its cofactors through the techniques of RNA extraction, cDNA synthesis, and PCR, eight genes showed the most methylation changes in ectopic and eutopic endometriosis tissues and organoids, which are *VAX1, ISL2, PITX2, BARX, LBX1, ARX, OTX2* and *EN1*(8, 9).

In recent years, many studies have been conducted on endometriosis and biomarkers related to the diagnosis of this disease. In 2010, Hakan Cakmak et al.'s studies on the molecular mechanisms of *HOX* genes in endometriosis led to the discovery of the role of the progesterone gene in the development of this disease and showed that changing the expression of the progesterone receptor or reducing its activity may lead to a decrease or lack of regulation. Progesterone response and decreased expression of genes that respond to it, including *HOX* genes, in eutopic endometrium. In human endometrium, the expression of *HOXA10* and *HOXA11* is driven by sex steroids, and the maximum expression occurs at the time of implantation in response to increased progesterone levels(10). In 2015, Mehdian et al. evaluated the expression of *MIF, CD74,* and *COX-2* genes in normal, ectopic, and eutopic endometrium during the menstrual cycle and evaluated the level of *MIF* in peripheral blood and concluded that the relative mRNA expression of *MIF, CD74,* and *COX- 2* in ectopic endometrium is significantly higher than eutopic and control endometrium. As a result, the expression of *MIF, CD74,* and *COX-2* during the menstrual cycle can play an essential role in reproduction, inflammation, and endometrial regeneration(11).

In 2015, Jahormi et al showed that some genes that are related to the *HOX* family and are among the key genes in the development of endometriosis. In this study, the expression profile of 84 genes from the HOX family related to various aspects of the formation of nerve fibers using The qRT-PCR method was investigated and the result of this investigation was the overexpression of genes that are involved in various aspects of the growth of sensory neurons in the tissue and signal transmission. Genes involved in pain, touch, and formation of GABA neurons (*VAX1, LBX1, LBX2,* and *PITX2*), dopaminergic neurons (*EN1* and *PITX3*), committed to cutaneous sensory neuron fate (*PAX3*)), chemosensory integration (*PHOX2b*)), promoting nerve formation (*DRGX, EMX1, PHOX2B* and *OTP*), different aspects of somatic motor neuron (*ISL1, 2*), neural migration in eutopic and ectopic tissue compared to the control group, which can cause the formation of new neural network, as well as the release of many free inflammatory mediators. It is caused by endometriosis lesions that can stimulate the sensory nerves and cause pain symptoms(12).

Also, in 2018, Jahormi et al., during the investigation of *HOX* genes, a significant increase in the expression of some genes of the *HOX C* and *HOX D* gene categories and a decrease in the expression of *HOX A* and *HOX B* paralogs except for *HOX A1* in ectopic endometriosis tissues. Against healthy endometrial tissue showed that the change in the expression level of these genes in the endometrium may play a role in the pathogenesis of endometriosis because the expression pattern of *HOX* genes sequentially from 3' to 5' along the anterior-posterior axis (AP) during Development is embryonic and genes 3 are expressed before genes 5(13).

In 2020, Hibaoui et al used biopsies of human primary endometrial cells and patient-derived IPSCs to produce endometrial organoids and study them for disease modeling, drug screening, and testing and benchmarking for new treatments. As a result, considering the progress In the context of biopsy-derived organoids and IPSCs, there is good reason to be optimistic that endometrial organoids will advance our understanding of the molecular and cellular mechanisms in endometriosis(6).

In 2020, during their bioinformatic studies on the endometrial tissue of endometriosis patients, Chitsazian and colleagues supported the role of *HOX* family genes in the development of endometriosis. Bioinformatics analysis of HOX genes and related genes in endometriosis showed that genes such as *VAX2, PHOX2B, LMX1B, DLX1,* and *SIX4* have significant changes in gene expression of endometriosis tissue samples. Accordingly, the functional study showed that significant genes are involved in developmental, neural, and metabolic pathways. Many of these genes were not previously reported as genes related to endometriosis(14).

In 2021, Esfandiari et al. identified pathways related to stem cell proliferation that had different expressions, which was consistent with previous reports for this pathway in endometriosis. It was also reported that *HOX* gene expression is regulated by estradiol and

progesterone hormones. Endocrine regulation of *HOX* gene expression is important for reproduction(8).

In 2021, Esfandiari et al. compared DNA methylation in the range of *HOX* genes and its cofactors in ectopic and eutopic endometriosis organoids and ectopic and eutopic endometriosis tissue with control tissue. A conserved pattern of methylation changes in endometriosis tissues and organoids for most of The studied genes (56 out of 84) was found to maintain epigenetic changes in endometriosis organoids. The role of *HOXA1* in cell proliferation, migration, and invasion has been reported in endometrial cancer(9).

In 2022, during three separate studies conducted by Mikaili, Bakhtiari and Karbalai, it was determined that the expression of *HOX* genes affecting endometriosis including *VAX1, EN1, ISL2, LBX1, BARX, PITX2, OTX2* and *ARX* genes in the peripheral blood plasma of patients In endometriosis, the level of mRNA expression showed a significant increase compared to the peripheral blood plasma of healthy people. According to these studies, peripheral blood plasma was collected from women with endometriosis at levels 3 and 4, and after plasma separation by centrifugation and RNA extraction with the help of a kit The synthesis of cDNA and the design of related primers for the expression of these genes were evaluated by qRTPCR method(15-17).

At Royan Research Institute, studies have been conducted on *HOX* family genes and their cofactors in patients with endometriosis, and the results of these studies showed that eight genes, including *VAX1, ISL2, PITX2, BARX, LBX1, ARX, OTX2,* and *EN1,* are the most They had the changes in people with endometriosis, and also these genes were significantly increased in the peripheral blood of these people, therefore, considering the inflammatory nature of these genes, in this proliferation, the expression of *HOX* family cofactor genes, including *BARX1, VAX1, ISL2,* and *OTX2* were investigated in follicular fluid of women with endometriosis and healthy women.

Materials and Methods

In this study, two groups of patients and controls were studied. The patient group (20 people) were infertile women with endometriosis who were treated with IVF/ICSI cycles at the Infertility Center of Royan Research Institute and were diagnosed with Endometriosis as the third or fourth type. After a complete review of the case of having a Doppler ultrasound to confirm the presence of endometriosis, these people were included in the study if they were approved by the doctor and met the criteria for entering the project.

The control group (20 people) were healthy women who were referred to the Infertility Center of Royan Research Institute for reasons other than endometriosis and were suffering from male factor in the field of infertility, either for egg freezing or to determine the gender of the fetus and donate eggs to the center. After a complete review of the case of having a Doppler ultrasound to confirm the absence of endometriosis, if approved by the doctor and having the criteria for entering the project, these people were included in the study.

Inclusion criteria include age 18-45 years, regular menstrual cycle (21-35 days), stage III or IV of endometriosis, agonist or antagonist protocol (GnRh) and HCG trigger or HCG trigger with double trigger and exclusion criteria include Endometrial cell changes such as hyperplasia and carcinoma, PCOS, DOR, herpes simplex type 2 (HSV-2), HPV and HSP and chocolate follicular fluid.

This study was conducted at Royan Research Institute. After receiving the informed consent from the studied women, their follicular fluid sample was taken from the operating room of the treatment unit during the ovulation procedure. Vials containing each person's follicular fluid were placed in liquid nitrogen (-196°C) for 120 seconds. They were frozen and immediately transferred to a -70°C freezer and used for genomic studies. RNA in follicular fluid was extracted by RNA extraction kit and cDNA was synthesized from RNA finally synthesized cDNA was evaluated by Real-Time PCR technique.

In this study, the SMOBIO kit was used for cDNA synthesis, and to increase the efficiency of the reaction, both types of random primer mix and Oligo (dT) primers were used.

To check the expression level of *BARX1, VAX1, ISL2,* and *OTX* genes, a real-time PCR technique was used from the cDNA samples obtained from different groups, and their expression was normalized to the expression of 18s internal control. The relative expression of studied genes was measured by comparing the expression level of these genes in patient and control groups using 18s internal control and ΔCT method. The CT number (Cycle Threshold) is the first significant increase in the PCR product, which is also called the threshold cycle. In short, the calculations were done in such a way that in each group, the CT of the examined genes in each sample was subtracted from the internal control CT of 18s in the same sample, and ΔCT was obtained for each group separately. The noteworthy point is the CT number of housekeeping genes, which should be less than 20-25 to indicate the good quality of cDNA.

In this study, the relative expression of the studied genes was measured by comparing the expression levels of these genes in the patient and control groups using the 18S internal control and the 2-ΔΔCT method. In summary. The calculations were done as follows:

(internal control) Ct - (gene of interest) ΔCt = Ct

(control sample) Ct Δ- (examined sample) Ct Δ ΔΔCt = t

The final result of the two patient and control groups was -ΔΔCT 2, which was analyzed using the SPSS

program and the t-test statistical test. A statistical significance level of 0.05 was also considered.

RESULTS:

In this study, 20 healthy people (as a control group) and 20 patients with endometriosis were included after considering the inclusion and exclusion criteria. The patient group had an average age of 32 ± 0.5 years, while the control group had an average age of 31.5 ± 0.7 years.

Examining the expression results of *HOX* family cofactor genes in follicular fluid

Independent T analysis was performed to compare gene expression in both control and patient groups. The results are as follows.

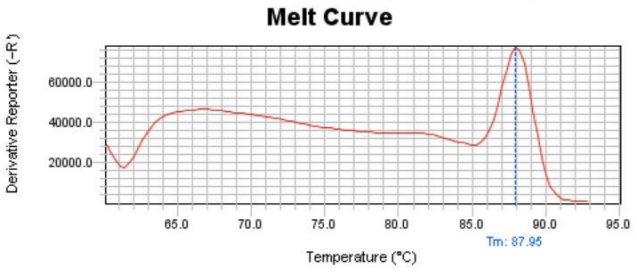
Table 1: Statistical comparison of the two studied groups

Standard Deviation	Average	number	Group	Gene
62000/187	6100/360	20	Control	*BARX1*
67630/25	6129/113	20	patient	
57103/291	1342/137	20	Control	*ISL2*
71657/1076	8707/356	20	patient	
84328/1737	9569/442	20	Control	*OTX2*
81378/174	2449/90	20	patient	
09590/4041	3317/844	20	Control	*VAX1*
90689/2085	2071/531	20	patient	

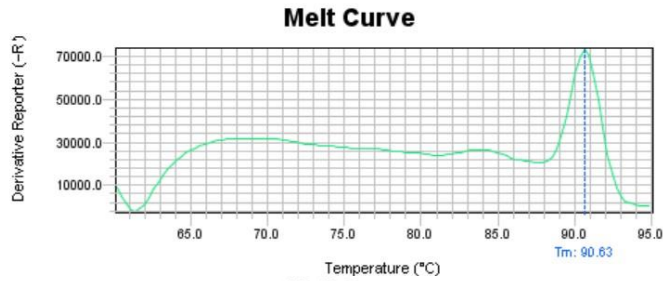
For the *BARX* gene, the assumption of equal variances was confirmed, with a T value of 0.881 and a significance level (p-value) of 0.004. For the *ISL2* gene, the assumption of equal variances was also confirmed, with a t value of -1.016 and a significance level of 0.024. For the *OTX* gene, the assumption of equal variances was accepted, with a t value of 0.924 and a significance level of 0.011. For the *VAX* gene, the assumption of equal variances was confirmed, with a t-value of 0.323 and a significance level of 0.003. These results indicate that for some genes, there are statistically significant differences between the control and patient groups.

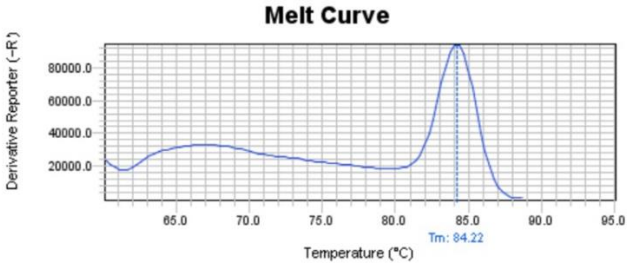
The melt curve of the Real-Time PCR product for the *VAX1, OTX2, ISL2,* and *BARX1* genes

In Figure 1, the melt curve of the Real-Time PCR product for the *VAX1, OTX2, ISL2,* and *BARX1* genes in the follicular fluid of patients with endometriosis is shown. For greater accuracy, each sample in each group was repeated twice:



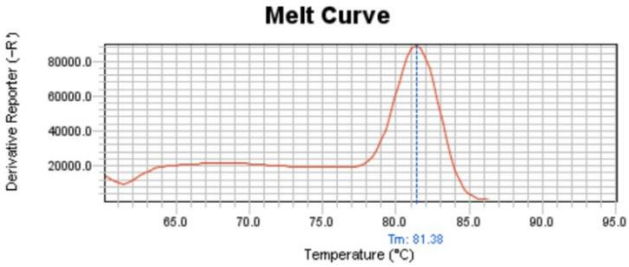
a





b

c



d

Chart 1: Melting Curve of Real-Time PCR Genes: a) *BARX1*, b) *VAX1*, c) *OTX2*, d) *ISL2*

Quantitative Results from the Analysis of Gene Expression of *OTX2*, *VAX1*, *ISL2*, and *BARX1* in Follicular Fluid of the Patient and Control Groups

The expression of the *OTX2* gene in follicular fluid samples from individuals with endometriosis was compared with that of the control group (Chart 2). The results indicate that the expression level of this gene in the follicular fluid of patients with endometriosis is significantly higher compared to the control group.

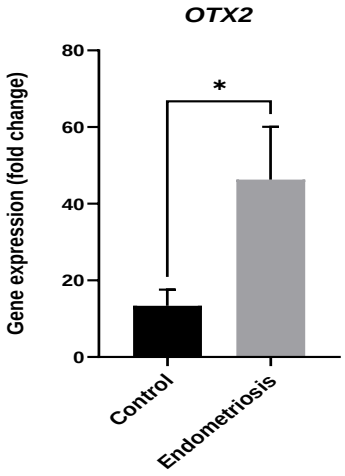


Chart 2: *OTX2* Gene Expression in Follicular Fluid of Endometriosis and Control Groups, The expression level of the *OTX2* gene in the follicular fluid of women with endometriosis showed a significant increase compared to healthy women (n=20, p-value=0.011). The significant difference is denoted at the level of (* p < 0.05), and the values are presented as mean ± SEM.

The expression of the *BARX1* gene in follicular fluid samples from individuals with endometriosis was compared with that of the control group (Chart 3). The results indicate that the expression level of this gene in the follicular fluid of patients with endometriosis is significantly higher compared to the control group.

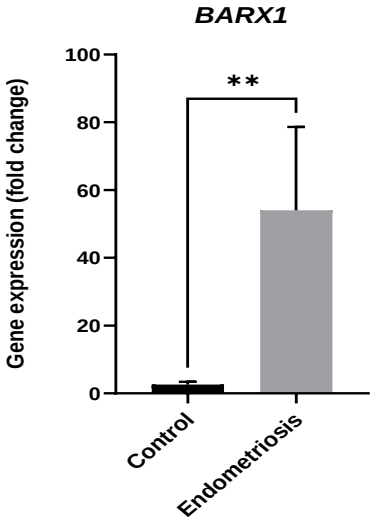


Chart 3: *BARX1* Gene Expression in Follicular Fluid of Endometriosis and Control Groups, The expression level of the *BARX1* gene in the follicular fluid of women with endometriosis showed a significant increase compared to healthy women (n=20, p-value=0.004). The significant difference is denoted at the level of (p < 0.01), and the values are presented as mean ± SEM.**

The expression of the *VAX1* gene in follicular fluid samples from individuals with endometriosis was compared with that of the control group (Chart 4). The results indicate that the expression level of this gene in the follicular fluid of patients with endometriosis is significantly higher compared to the control group.

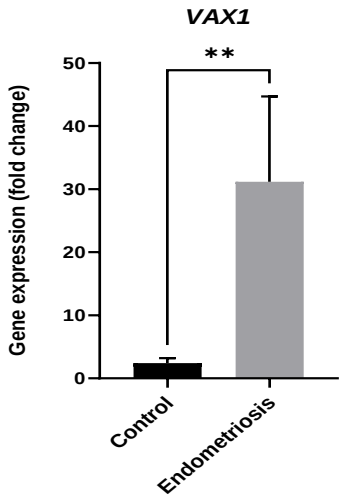


Chart 4: *VAX1* Gene Expression in Follicular Fluid of Endometriosis and Control Groups, The expression level of the *VAX1* gene in the follicular fluid of women with endometriosis showed a significant increase compared to healthy women (n=20, p-value=0.003). The significant difference is denoted at the level of (p < 0.01), and the values are presented as mean ± SEM.**

The expression of the *ISL2* gene in follicular fluid samples from individuals with endometriosis was compared with that of the control group (Chart 5). The results indicate that the expression level of this gene in the follicular fluid of patients with endometriosis is significantly higher compared to the control group.

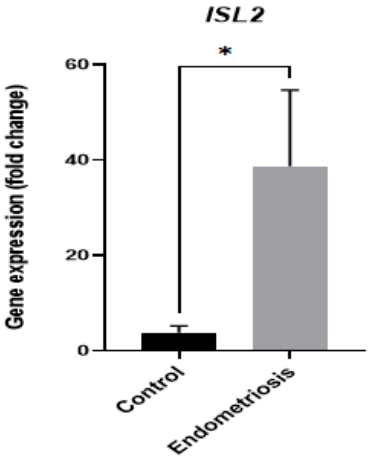


Chart 5: *ISL2* Gene Expression in Follicular Fluid of Endometriosis and Control Groups, The expression level of the *ISL2* gene in the follicular fluid of women with endometriosis showed a significant increase compared to healthy women (n=20, p-value=0.02). The significant difference is denoted at the level of (* p < 0.05), and the values are presented as mean ± SEM.

The results of gene expression analysis in the two study groups indicate significant differences in the mean and standard deviation between the patient and control groups. In the statistical analysis section, independent T-tests were employed to compare gene expression levels, revealing significant differences for certain genes, such as *BARX1*, *OTX2*, *ISL2*, and *VAX1*. These differences are reported along with the corresponding T values and significance levels, indicating notable statistical differences between the patient and control groups. Based on these results, it can be concluded that the studied factors have different effects on gene expression in individuals from different groups, and these differences are significantly reflected in the obtained data.

DISCUSSION:

The results obtained from the Real-time PCR method indicated that the expression of these genes in the follicular fluid of women with endometriosis has significantly increased. This increase in expression may indicate a key role in the inflammatory processes associated with endometriosis, which in turn affects the quality of the oocytes and fertility success. In individuals with endometriosis, the immune system is severely impaired, leading to the spread and progression of endometriosis. In these individuals, the levels of active macrophages and cytokines, including inflammatory interleukins (IL-6, IL-8, IL-4) and tumor necrosis factor-alpha (TNF-α), increase in the peritoneal fluid (18).

Numerous studies have focused on the role of the *HOX* gene family and their cofactors in the growth and development of the female reproductive system. Previous research has shown that *HOX* genes such as *HOXA10* and *HOXA11* are strongly associated with the development of the uterus and oocytes(13). Research has shown that alterations in the expression of *HOX* family genes can lead to reproductive anomalies such as endometriosis(19). Other research has reported that disruptions in the genes *HOXD3, HOXA11, HOXB3,* and *HOXA10* play a role in the development of endometriosis(20, 21).

HOX gene cofactors are proteins that specifically interact with *HOX* genes and play a key role in regulating gene expression. These cofactors help *HOX* genes accurately control the location and timing of target gene expression, which is vital for proper tissue growth and development. The results of this research are consistent with previous studies confirming that *HOX* family genes and their cofactors play an important role in the development of endometriosis through a series of signaling pathways. Continuing studies at Royan Institute, Esfandiari and colleagues examined the expression of *HOX* family genes and their cofactors in ectopic endometriosis tissue and organoids. The results

showed that the expression patterns of *HOXA3, HOXA4, HOXA9, HOXB2, HOXB3, HOXB8, HOXC12, HOXC8, HOXD1, HOXD3, HOXD9, HOXD11, HOXD12, HOXD13, ALX1, ARX, BARX1, CDX1, DLX1, DLX2, PITX2T, LBX1, LBX2, LMX1, OTX2, ISL1, EN2,* and *EMX1* significantly increased in endometriosis tissue of individuals with endometriosis compared to healthy individuals. Among them, 8 genes *VAX1, ISL2, PITX2, BARX1, LBX1, ARX, OTX2,* and *EN1* showed the most significant changes in women with endometriosis(9, 22). The significant increase in the expression of the *VAX1, ISL2, BARX1,* and *OTX2* genes observed in our study aligns with previous findings. These genes act as key factors in the inflammatory signaling pathways, contributing to inflammation and tissue damage in endometriosis(23), Because these genes exhibited significant changes in individuals with endometriosis, the signaling pathways might be related to apoptotic pathways in granulosa cells. This leads to the destruction of granulosa cells and increased expression of *HOX* family cofactors, including *ISL2, VAX1, BARX1,* and *OTX2* in the follicular fluid of women with endometriosis. Similarly, our results indicate that the increased expression of these genes in follicular fluid may be associated with elevated levels of inflammation.

Given the research results, the relationship between their increased expression and endometriosis is explored further. The *VAX1* gene plays a crucial role in the differentiation and development of embryonic cells. An increase in *VAX1* gene expression in the follicular fluid of endometriosis patients can lead to the activation of signaling pathways associated with abnormal differentiation of endometrial cells, which can facilitate the proliferation and survival of endometriosis cells outside the uterus. Additionally, this gene likely plays a role in creating a favorable environment for the survival of endometriosis cells and their resistance to apoptosis through the improper regulation of specific transcription factors, potentially creating an unsuitable environment for ovulation and oocyte quality. Moreover, the increased expression of *VAX1* in follicular fluid may disrupt oocyte maturation processes, which in turn can negatively affect its quality(24, 25, 26).

In this study, the *BARX1* gene showed higher expression in patients with endometriosis compared to the control group. The role of this gene in endometriosis is characterized by its impact on pathways related to cell proliferation and invasion. Increased expression of *BARX1* may be associated with the enhanced ability of endometrial cells to migrate to areas outside the uterus and grow in new environments(27).

The increased expression of the *OTX2* gene in endometriosis indicates a disruption in the regulation of normal cell growth and differentiation. This gene, primarily active in embryonic development stages, might be associated with the abnormal proliferation and differentiation of endometrial cells in endometriosis. It may lead to uncontrolled cell proliferation and resistance to natural regulatory mechanisms by activating cell proliferation-related signaling pathways(28, 29).

The *ISL2* gene plays a role in the development of the nervous and muscular systems. Overexpression of this gene in endometriosis patients may be associated with the misregulation of factors related to cell migration and invasion, including matrix metalloproteinases (MMPs), contributing to the spread of endometriosis tissues. Additionally, this gene, through its effects on signaling pathways related to inflammation and angiogenesis, can exacerbate inflammatory responses and increase the levels of inflammatory cytokines(30, 32).

Conclusion

Endometriosis is a chronic inflammatory disease that can significantly affect the female reproductive system. The results of this study showed a significant increase in the expression of VAX1, ISL2, BARX1, and OTX2 genes in the follicular fluid of women with endometriosis, which seems to activate key signaling pathways related to cell growth, proliferation, migration, and inflammation, thus contributing to the pathogenesis and spread of ectopic endometrial tissues. This can also disrupt the natural oocyte maturation processes. These genetic changes may not only affect oocyte quality but also reduce the chances of pregnancy. Therefore, focusing on the impact of inflammation on oocyte quality can be crucial in developing new therapeutic methods aimed at reducing inflammation and improving oocyte quality.

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