

Semi-Quantitative Analysis of *HOXA11*, Leukemia Inhibitory Factor and Basic Transcriptional Element Binding Protein 1 mRNA Expression in the Mid-Secretory Endometrium of Patients with Endometriosis

Zohreh Alizadeh^{*1}, Naser Shokrzadeh², Massoud Saidijam¹ and Marzieh Farimani Sanooee³

¹Research Center for Molecular Medicine, Hamadan University of Medical Sciences, Hamadan, ²Dept. of Anatomy, Gonabad University of Medical Sciences, Gonabad, Khorasan Razavi, ³Dept. of Obstetrics and Gynecology, School of Medicine, Hamadan University of Medical Sciences, Hamadan, Iran

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ABSTRACT

Background: *HOXA11* and leukemia inhibitory factor (*LIF*) and basic transcriptional element binding protein1 (*BTEB1*) are expressed in endometrium throughout the menstrual cycle and show a dramatic increase during the mid-luteal phase at the time of implantation. In this case-control study, the expression pattern of these mRNA was evaluated in patients with endometriosis at the time of implantation. We also describe a semi-quantitative RT-PCR protocol optimized in our laboratory. **Methods:** Eight patients with endometriosis were considered as our case and 8 fertile women as control group. Expression levels of *HOXA11*, *LIF* and *BTEB1* mRNA were measured in endometrium during the mid-secretory phase using semi-quantitative RT-PCR. **Results:** We describe the detailed procedure for the analysis of *HOXA11*, *LIF* and *BTEB1* mRNA levels. Endometrial *HOXA11* and *LIF* mRNA expression levels (normalized to β -actin expression) were significantly decreased in endometrium of infertile patients with endometriosis compared with healthy fertile controls at the time of implantation ($P<0.05$). A similar trend was seen in *BTEB1* mRNA expression. **Conclusion:** The results suggest that the alteration in expression pattern of the some genes could account for some aspects of infertility in endometriosis. *Iran. Biomed. J.* 15 (3): 66-72, 2011

Keywords: Endometriosis, Implantation, *HOXA11*, Leukemia inhibitory factor (*LIF*), Basic transcriptional element binding protein1 (*BTEB1*)

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 the normal location and is mainly associated with infertility. It affects approximately 10-15% of women of reproductive age and 25-50% of all women with infertility.[1].

During the menstrual cycle, the ovarian steroid hormones, estrogen and progesterone control a dramatic transcriptional reprogramming of endometrial cells, leading to a receptive state for blastocyst implantation and the establishment of pregnancy [2]. Optimal implantation conditions are preceded by proliferation and differentiation of elements, including endometrial glands, stroma, blood vessels, smooth muscle cells and fibroblasts. Impaired endometrial growth and differentiation may be a significant factor

contributing to infertility [2].

Several genes have been identified that are essential for preparing endometrium to receive embryo, like *HOXA11*, leukemia inhibitory factor (*LIF*) and basic transcriptional element binding protein1(*BTEB1*) [3].

HOXA-11 homebox genes are one of the best-known transcription factors participating in implantation [4]. In the mid-secretory phase of a menstrual cycle, which is coincide with the time of implantation, *HOXA11* mRNA expression is up-regulated in both endometrial glandular and stromal cells in women [3, 4]. Findings regarding the importance of *HOXA-11* gene are contradictory. Some authors have reported a dramatic rise in the *HOXA-11* expression level in the mid-luteal phase [4, 5], whereas other authors do not confirm this phenomenon [3].

LIF expression is essential for embryo-endometrium interaction and for blastocyst implantation in mice and humans [6]. In humans, *LIF* mRNA and protein levels

*Corresponding Author; Tel.: (+98-811) 8380 579; Fax: (+98-811) 8380 208; E-mail: alizadeh@umsha.ac.ir

are low in the proliferative phase, and expression markedly increases in the secretory phase at the time of implantation [7]. High LIF expression is an indicator of receptive endometrium in fertile women. However, in infertile individuals, the data on endometrial LIF expression and secretion are controversial [8].

BTEB1 is an endometrial transcription factor that may play a role in regulation of endometrial cell growth by modulating gene transcription [9]. This Kruppel-like family member gene directly interacts with the progesterone receptor to mediate progesterone-responsive gene expression in endometrial cells [3]. Gene array studies have established that there are aberrantly expressed genes in endometrium of women with endometriosis compared to the women without endometriosis during the implantation window [10]. But expression pattern of *BTEB1* in endometriosis has not been previously examined in human, while one study examined the expression of this mRNA in mice model [11].

The analysis of assisted reproductive outcomes revealed that women with endometriosis and infertility who undergo *in vitro* fertilization and embryo transfer have pregnancy rates that are about 50% of women who undergo *in vitro* fertilization and embryo transfer for tubal factor infertility [1]. Abnormalities in the endometrium resulting in failure of embryonic implantation are believed largely to account for the lower pregnancy rates in women with endometriosis. However, the underlying mechanisms of endometriosis-associated infertility are remained unclear [12]. Many factors may be involved, and studies suggest that endometrial molecular defects, involved in implantation during the implantation window, might be the cause of endometriosis-associated infertility [10, 12]. Findings regarding the importance of *HOXA-11*, *LIF* and *BTEB1* genes in endometriosis are contradictory.

The aim of our study was to determine whether endometrial expression of individual selected genes *HOXA11*, *LIF* and *BTEB1*, which are crucial for implantation, is normal in women with endometriosis infertility. Here, we have also discussed a semi-quantitative RT-PCR approach, which is inexpensive, reliable, and adaptable to different laboratories to compare the expression level of considered genes in endometrium of patients with endometriosis with that in normal fertile controls.

MATERIALS AND METHODS

Patients. In this research, 8 women with endometriosis were studied. 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 device for at least 6 months prior to surgery. Exclusion criteria were bilateral tubal occlusion. All included patients were based on visualization of endometrial lesions found during laparoscopy. The patients with moderate (stage I to III) endometriosis, according to the revised American Fertility Society endometriosis staging (Canis *et al.* [13]) were considered for further studies. In addition, endometrial tissues were obtained from 8 healthy fertile women with macroscopically normal pelvic cavities as control. Endometrial tissue biopsies were performed during 19-23 days of a menstrual phase using an endometrial suction catheter. Each sample was divided into two portions. The first tissue portion was fixed in 10% formalin for histopathological examination. The second portion was immediately collected in RNA extraction solution (RNX-Plus, Cinagene Company, Iran) and stored at -80°C until further analysis was performed. All tissue samples were obtained with full and informed patient consent. The research protocol was approved by the Medical Ethics committee of Hamadan University of Medical Sciences.

RNA extraction. To obtain total RNA, each sample was placed in 1 mL of RNA extraction solution (RNX-Plus, Cinagene Company, Iran) and homogenized by shaking. The cellular lysate was incubated, chloroform 0.2 mL was added, and the samples were centrifuged (17,000 ×g centrifugal force at 4°C for 15 min). The clear, aqueous phase was collected and transferred to a new tube, and RNA was precipitated with isopropanol and washed with 75% ethanol. The RNA pellet was air-dried, then resuspended with RNase-free water [14]. From all obtained RNA samples, 2 µl was analyzed using the Epoch Microplate spectrophotometer (BioTek, USA).

Reverse transcription. Single-stranded cDNA was synthesized using RivertAid™ First Strand cDNA Synthesis Kit (Fremontas, Canada) using 4 µg of RNA, according to the manufacturer's protocol. The transcription process included incubation of the reaction mixture at 42°C for 60 min, followed by 5 min at 70°C. The cDNA was stored at -80°C until further use for PCR.

Polymerase chain reaction. Primer pairs for the amplification of cDNA coding for *HOXA11*, *BTEB1* and *LIF* were designed from the GenBank databases using the AlleleID 6 software and checked for minimum overlap. The sequences of primers used in the experiments are presented in Table 1. PCR were carried out using Mg⁺² (3 mM), Taq-polymerase

Table 1. Primer sequences used in PCR.

Accession no.	Primer sequence	Product size (bp)	Gene
<i>HOXA11</i>	412	Sense: 5'-TTCCAGCACCACACTCAG-3' Anti-sense: 5'-AGCATTTCCCTAACTCTTTCC-3'	NM_021192.2
<i>LIF</i>	710	Sense: 5-TTGAAGTGTGCTGTGAAC-3' Anti-sense: 5'-GGAAGAGAACGAAGAACC-3'	NM_002309
<i>BTEB1</i>	638	Sense: 5'-TGGTCTCCTTCCTGTGTTC-3' Anti-sense: 5'-TAGTGATGGCTGTTGTATTGG-3'	NM_001206.2
<i>β-actin</i>	360	Sense: 5'-CGTACCACTGGCATCGTGAT-3' Anti-sense: 5'-GTGTTGGCGTACAGGTCTTTG-3'	NM_001198842

(2 unit), PCR buffer, dNTP (200 μ M) and a pair of specific primer [10 pmol/μ] in a final volume of 30 μl each tube. The PCR conditions were as follows: initial denaturation at 95°C for 2 min followed by 32 cycles for both *LIF* and *BTEB1* and 29 cycles for *HOXA11* and 26 cycles for β-actin [as control] of denaturation at 95°C for 30 s, annealing at 50°C for *HOXA11*, 58°C for *LIF*, 48°C for *BTEB1* and 49°C for β-actin for 30 s, and extension at 72°C for 1 m and final extension at 72°C for 7 m. The expected length for PCR products is given in Table 1. PCR for each sample was duplicate.

Optimizing PCR conditions for semi-quantitative analysis. For semi-quantitative analysis, the PCR must be in its exponential range providing doubling of the products amount with each cycle [15, 16]. To define the exponential range, several independent PCRs were performed where 3.5 μl of the reaction mix was taken out every determined cycle. The PCR products were loaded on a 1% agarose gel and evaluated for the relative increase in the PCR product obtained with each cycle.

Gel electrophoresis. The PCR products of each interested gene and β-actin were loaded on to the same ethidium bromide-stained agarose gels (1%). A 1-kb DNA ladder molecular weight marker (Fermentas, Canada) was run on each gel to confirm expected molecular weight of the amplification product. Stained gels were recorded and the band intensity was evaluated using the TotalLab TL 120 2009 software. Band intensity was expressed as relative absorbance units. The ratio between the sample RNA to be determined and β-actin was calculated to normalize for initial variations in sample concentration and as a control for reaction efficiency. Mean and standard deviation of all experiments performed were calculated after normalization to β-actin [15, 16].

RESULTS

All samples underwent histological evaluation, and normal mid-secretory phase of endometrium was identified. Endometrium from subjects with endometriosis and controls was evaluated for mRNA expression of *HOXA11*, *BTEB1*, and *LIF*.

For quantification of gene expression by semi-quantitative RT-PCR, PCR reactions must be carried out during the exponential (log) phase. Results of kinetic analysis by amplifying PCR products over different number of PCR cycles are shown in Figure 1. The optimal numbers of PCR cycles determined were 29, 32, 32 and 26 for *HOXA11*, *LIF*, *BTEB1* and β-actin, respectively (Fig. 1).

Two samples to be compared, obtained from endometrium of control and patients with endometriosis, were amplified for consecutive cycles within the exponential phase of PCR reaction.

Compared with controls, endometrial *HOXA11* mRNA expression (normalized to β-actin expression) was significantly decreased in biopsies from endometriosis (10.42 ± 1.45 and 48.95 ± 4.23 , respectively; $P < 0.05$; Fig. 2).

Endometrial *BTEB1* mRNA expression (normalized to β-actin expression) was significantly lower in uteri with endometriosis than controls (11.06 ± 0.70 and 50.57 ± 1.93 , respectively; $P < 0.05$; Fig. 2).

Analysis of endometrial *LIF* mRNA expression demonstrated a similar trend between uteri with endometriosis and controls (10.88 ± 1.18 and 40.43 ± 0.27 , respectively; $P < 0.05$; Fig. 2).

DISCUSSION

Endometriosis is associated with poor reproductive outcomes [10, 12]. Although defective implantation is likely due to an endometrial defect, no specific endometrial deficiency has been identified that would explain these clinical findings. Histological

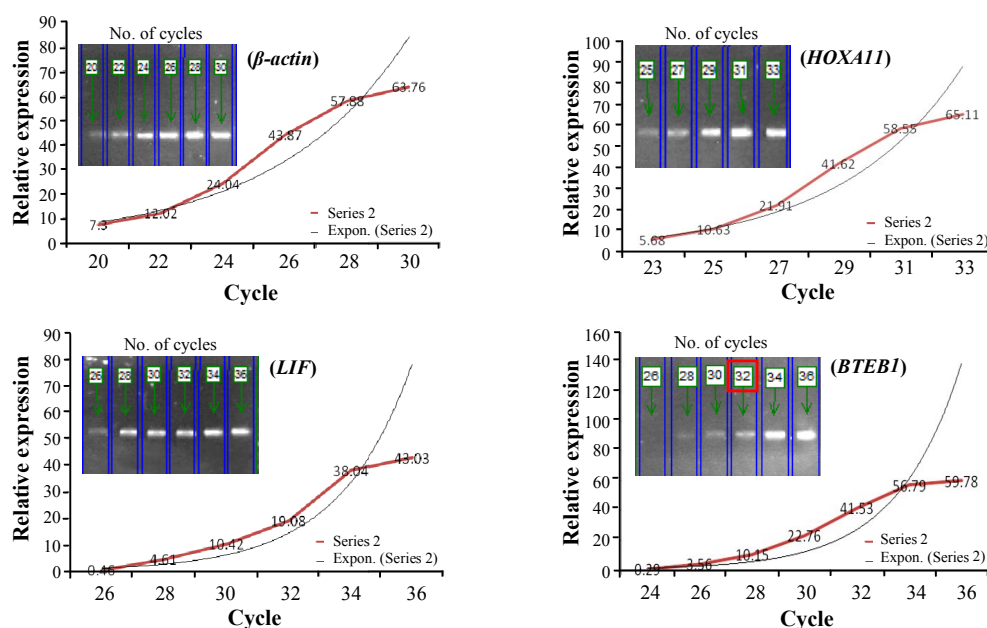


Fig. 1. Determination of the exponential range of amplification. Log (peak areas) plotted against number of PCR cycles. Details of the original gels are shown inside the blots.

characterization of endometrium from uteri with endometriosis revealed no consistent endometrial abnormality [17]. Histology alone cannot effectively assess endometrial receptivity; molecular evaluation of the endometrium is a potential means of identifying defects in receptivity. Alterations in endometrial expression of some genes have been identified in several clinical conditions associated with impaired endometrial receptivity: myomas and hydrosalpinges [7, 18].

In this study, the effect of endometriosis on the endometrium was evaluated using several established molecular markers of endometrial receptivity: *HOXA11*, *LIF* as well as *BTEB1*.

For quantification of mRNA levels, reliable methods are necessary to investigate the gene expression. There are three RT-PCR based methods for relative quantification of mRNA: semi-quantitative, competitive and real-time RT-PCR. Breljak *et al.* [19] compared these methods and showed that all of them gave reliable and comparable results. Thus, in spite of rapid advances made in the area of real-time RT-PCR, end-point RT-PCR such as semi-quantitative RT-PCR may still remain useful techniques for relative mRNA quantification.

In this study, by using semi-quantitative RT-PCR, we have compared the expression of interested mRNA in endometrium of patient with endometriosis and normal fertile women during mid-secretory phase of menstrual cycle. Although semi-quantitative RT-PCR allows quantification of mRNA level and requires only common laboratory equipment, one of the major

drawbacks of this method is the need for determination of exponential phase of the PCR reaction for each gene to be quantified [15, 17].

The prerequisite for a PCR to be used for quantification is that PCR product is measured within the exponential phase of the PCR reaction, where the amount of amplified target is directly proportional to the input amount of target. Therefore, quantification of gene expression by semi-quantitative RT-PCR must be carried out during the exponential (log) phase of the PCR reaction and plateau phase must be avoided [19]. Exponential phase of the PCR reaction can be determined by amplifying equivalent amounts of cDNA over different numbers of PCR cycles or by amplifying dilutions of cDNA over the same number of PCR cycles [16, 17]. In this work, we performed kinetic analysis by amplifying PCR products over different numbers of PCR cycles and showed that PCR reaction was exponential between cycles 26 and 32. Subsequently, kinetic analysis was performed within the given range of cycles. Similar kinetic analysis was also done for β -actin (a housekeeping gene as control). Because internal control RNA are typically expressed housekeeping genes of high abundance, their amplification shows exponential phase with few PCR cycles [16]. In this study, the cycle for β -actin was lower than others (26 cycles).

During the menstrual cycle, the ovarian steroid hormones, estrogen and progesterone, control a dramatic transcriptional reprogramming of endometrial cells, leading to a receptive state for blastocyst implantation and the establishment of pregnancy [3].

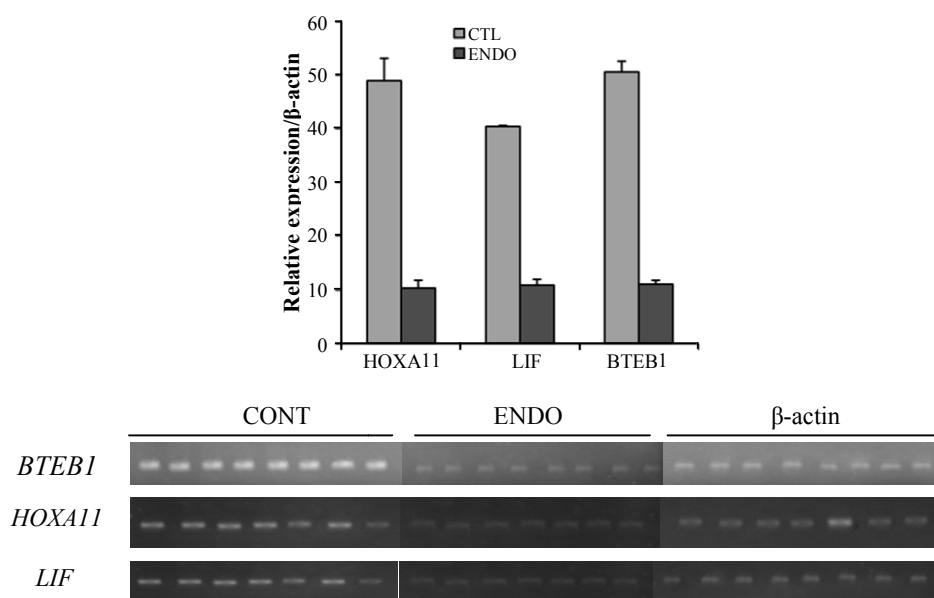


Fig. 2. Mean endometrial *HOXA11*, *BTEB1* and *LIF* mRNA expression (normalized to β -actin). Endometrium was sampled from uteri with endometriosis (ENDO) and compared with control groups (CONT). Details of the original gels are shown on the bottom of the blot.

Several genes have been identified that are essential for decidualization and changes of endometrial cells in response to hormones, including the *HOXA11*, *BTEB1* and *LIF* [3].

Homeobox (Hox/HOX) genes encode transcription factors that mediate embryonic development. In the human, *HOXA11* is expressed in endometrial glands and stroma throughout the menstrual cycle [4, 20]. *HOXA11* have been demonstrated to be necessary for implantation in mice. Targeted disruption of this gene result in sterility. The knock-out mice ovulate, but their embryos do not implant in their uterus. Wild-type embryos also do not implant in the knock-out mice; however, knock-out mouse embryos implant in a wild-type uterus. This targeted disruption results in an endometrial defect, not an embryo defect, in which implantation is severely altered [21]. Similarly a defect in HOX expression in patients with endometriosis may lead to a decrease in implantation without an appreciable pathology noted on histological examination. We found statistically significant lower *HOXA11* transcript levels in infertile women compared to the control group. Our results were similar to those reported by Szczepańska et al. [20] and Taylor et al. [22]. Their findings also suggest that infertility in endometriosis group of patients may have its source in the altered endometrium function, i.e. lack of its optimal receptivity.

Some of the key players in the receptivity of the endometrium are cytokines belonging to IL-6 superfamily, namely LIF and IL-11 [23]. An important role for *LIF* in implantation was shown on *LIF*

knockout mice, when embryo implantation did not occur. In endometrium of healthy women, LIF and *LIF* mRNA are expressed throughout the menstrual cycle [24]. Also, it has been shown that infertile women exhibited low levels of LIF in endometrial flushing and immunohistochemical staining [23]. In contrast, other studies report no change in endometrial *LIF* mRNA or secretion by endometrial biopsies from infertile compared to fertile women [25]. In the present study, endometrial *LIF* mRNA expression was significantly decreased in biopsies from endometriosis. This result is the same with Dimitriadis et al. [25] and disagrees with those observed by Mikolajczyk et al. [23]. Dimitriadis et al. [25] showed that LIF immunostaining intensity in glandular epithelium was significantly lower in endometrium from women with endometriosis compared to controls. According to Mikolajczyk et al. [23], LIF in uterine flushing shows a lower level compared with controls; however, the differences failed to reach statistical significance. Their results were confirmed by assessment of *LIF* mRNA in RT-PCR reactions. This difference with our results could be two possible explanations. In our study, the number of patients was lower compared with study of Mikolajczyk [23]. Also, we have used endometrium from women with stage I-III and they used stage I/II. Nevertheless, our study is generally in agreement with their paper. In some patients with endometriosis, there might be a decreased secretion of LIF, but this defect is not a common feature of patients with endometriosis.

The present study indicates the possibility that

endometrium of some infertile women with endometriosis has abnormalities in expression of *LIF* which may contribute to altered uterine receptivity, resulting in infertility. Interestingly, mice treated with a single intraperitoneal injection of peritoneal fluid from infertile women with endometriosis had decreased implantation sites and a reduction in endometrial *LIF* mRNA compared to treatment with peritoneal fluid from control women [27]. This suggests that environmental factors could also influence genes expression regulation.

BTEB1 is an endometrial transcription factor that may play a role in regulation of endometrial cell growth by modulating gene transcription [9]. Targeted mutation in *BTEB1* has been shown to result in subfertility and uterine hypoplasia [28]. Lee and Taylor [11] created experimental endometriosis in mice and examined the expression of *BTEB1* in the eutopic endometrium; however, we should be very careful about using the results of these studies in mice onto human subjects. We examined the expression of this gene in human endometriosis and detected a significant decrease in endometrial *BTEB1* expression of mRNA in infertile patients with endometriosis in compare with normal fertile controls.

Uterine receptivity and implantation are complex processes requiring the co-coordinated expression of molecules by the embryo and uterus during implantation. *HOXA11* and *LIF* and *BTEB1* are expressed in different kinds of endometrial cells throughout the menstrual cycle and show a dramatic increase during the mid-luteal phase at the time of implantation in response to estrogen and progesterone.

This study has identified lower expression of *HOXA11*, *LIF* and *BTEB1* in women with endometriosis that may result in inadequate preparation of a receptive endometrium.

However, *HOXA11*, *LIF* and *BTEB1* mRNA are not the only molecules responsible for successful implantation [10]. 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. Further studies are needed to investigate other endometrial molecular defects during the window of implantation in infertile patients with endometriosis.

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