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Increased Gene Expression of *LITAF, TNF-α* and *BCL6* in Endometrial Tissues of Women with Endometriosis: A Case-Control Study

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Abstract

Objective: Endometriosis, as a common inflammatory chronic disease is characterized by endometrial tissue growth outside the uterine cavity. It was reported that lipopolysaccharides (LPS) activate a transcription factor called LPS-induced tumor necrosis factor-alpha (LITAF) in macrophages, which induced transcription of cytokine genes such as tumor necrosis factor alpha (*TNF-α)*. B-cell lymphoma 6 protein (BCL6) is a transcription factor which expression was increased in endometrial tissues of infertile women with endometriosis. In addition, it was shown that mRNA and protein of LITAF and BCL6 were inversely related in mature B lymphocytes and B-Cell lymphomas. Accordingly, we investigated gene expression of *LITAF, BCL6* and *TNF-α* in eutopic and ectopic endometrial tissues of women with endometriosis compared to the controls.

Materials and Methods: In this case-control study, 10 women with endometriosis (endometriosis group) and 10 women without endometriosis (control group) enrolled after diagnostic laparoscopy. Real-time polymerase chain reaction (PCR) technique was used to quantitatively analyze gene expression. One-Way ANOVA was used for data analysis.

Results: This study showed that *LITAF* gene expression was significantly higher in ectopic endometrial tissues compared to the control samples. Expression level of *BCL6* gene was significantly increased in the ectopic tissues of women with endometriosis compared to the control and eutopic samples. Although *TNF-α* gene expression was increased in the ectopic lesions compared to the eutopic and control endometrial samples, these differences were not significant.

Conclusion: The results suggested that over-expression of these inflammatory genes in ectopic endometrial lesions can be considered as a molecular scenario in pathophysiology of endometriosis by induction of inflammatory cascades and cellular proliferation.

Keywords: B-Cell Lymphoma 6 Protein, Ectopic, Endometriosis, LITAF, Tumor Necrosis Factor-Alpha

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Introduction

Endometriosis is an inflammatory chronic disease and it is diagnosed in women at reproductive ages (1). In this disease, endometrial tissues implant and grow outside the uterine cavity including fallopian tubes, ovaries, or in the pelvic cavity. The common symptoms of endometriosis are pain and infertility (2). The cause of endometriosis remains unclear, but previous studies have suggested that several factors are involved in its pathogenesis, including hormonal, genetic, and immune changes (3-5).

The immune system plays an important role in the beginning and progression of endometriosis. In particular, immune cells of innate and acquired immune system, play a key role in the survival and proliferation of endometrial cells outside the uterine cavity (6). In endometriosis, inflammation and alteration of immune cells functions are

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observed in the peritoneal cavity. It was also reported that the number of activated macrophages in the peritoneum was increased in women with endometriosis. In addition, macrophages secreted a variety of cytokines and growth factors (7). The cytokines that were elevated in endometriosis included interleukin 1 (IL-1), (8), thymic stromal lymphopoietin (TSLP), IL-10 (9), IL-6, IL-13, tumor necrosis factor-alpha (TNF-α) (10) and interferon-gamma (IFN-Ɣ) (11).

TNF-α is an inflammatory cytokine produced by macrophages, langerhans cells, microglia, astroglia, and Kupffer cells. *TNF-α* gene is located on chromosome 6 (6p21.33) containing four exons and three introns (12). Richter et al. (13), studied the peritoneal fluid of 65 women with endometriosis at advance stages according to revised American Fertility Society (rAFS) categories compared to 35 women of the control group. They showed that TNF-α secretion of macrophages was significantly higher in endometriosis group.

Lipopolysaccharide (LPS) in the outer membrane of gram-negative bacteria activates monocytes and macrophages to produce cytokines such as TNF-α, IL-1, and IL-6. Then, LPS binds to toll-like receptors (TLRs) on the membrane of macrophages and monocytes; thus presence of the microbial invaders is detected in the body (14). LPS activates a transcription factor called LPS-induced TNF-α (LITAF) which results in transcription of *TNF-α* gene (15). LITAF is a transcription factor that binds to a specific site of the TNF-α promoter leading to transcription of this gene (16). According to national center for biotechnology information (NCBI) gene database, location of *LITAF* gene is on chromosome 16 (16p13.13). This gene has 13 exons and 12 introns. Chung et al. (17) studied LITAF expression in the eutopic tissues of 11 women with endometriosis and 5 normal women by immunohistochemistry technique. They showed increased expression of the *LITAF* gene and protein in the eutopic tissues of endometrial women compared to the controls.

On the other hand, B-cell lymphoma 6 (BCL6) is a transcriptional inhibitor associated with cell proliferation (18). According to NCBI gene database, *BCL6* gene is located in the long arm of chromosome 3 (3q27.3) containing 12 exons and 11 introns. It was shown that the *BCL6* gene expression level was increased in endometrial tissues of women with endometriosis compared to the control group (19). In addition, it was observed that LITAF expression was inversely correlated with BCL6 expression in mature B lymphocytes and B-cell lymphomas (20). Inhibition of BCL6 transcription by LITAF in B lymphocytes suggested that LITAF may play a role in the development of mature B lymphocytes. After LITAF moving to the nucleus, it binds to the *BCL6* promoter. This leads to a decrease in the expression of *BCL6*. Inhibition of *BCL6* by LITAF occurs not only through transcriptional repression, but also partly through protein-protein interaction. Aberrant expression of LITAF can lead to BCL6 suppression and apoptosis in B cell non-Hodgkin lymphoma (B-NHL) from the mitochondrial pathway (21).

Although expressions of *BCL6* and *LITAF* have been studied in the eutopic endometrial tissues of women with endometriosis, relationship between these gene expressions in endometriosis, especially the ectopic tissues has not been investigated. Therefore, the aim of this study was to investigate gene expression levels of *LITAF*, *TNF-α*, and *BCL6* in the eutopic and ectopic endometrial tissues of women with endometriosis in comparison with the normal endometrial samples.

Materials and Methods

Subjects

In this case-control study, 20 women who underwent laparoscopic surgery because of chronic pelvic pain or infertility with unknown etiology were enrolled. According to laparoscopy findings, 10 women with endometriosis (endometriosis group) and 10 women without endometriosis (control group) were included women with endometriosis were in stages III and IV of disease according to the revised American Society for Reproductive Medicine (rASRM). rASRM classification defined four stages as minimal, mild, moderate and severe, which were determined based on the size of endometriosis lesions and adhesions in the peritoneum, ovaries and fallopian tubes (22).

The women were between 20 and 45 years old, and they had regular menstrual cycles, while they had not received any hormone medication in the last three months. These women had no other endometrial diseases such as endometrial hyperplasia and carcinoma and no inflammatory, autoimmune, endocrine diseases, and cancers.

Ectopic tissues were collected during laparoscopy. Eutopic tissues of women with endometriosis and endometrial tissues of the control group were collected by pipelle. Endometrial tissue samples were transferred into 2-ml-cryovialtubes (Greiner BioOne, Germany) with RNA later (Ambion, UK) and then frozen in liquid nitrogen (-196°C) for 30 seconds. They were then immediately stored at -80°C until it was used for genomic study.

Perl Primer and Gen Runner software were used to design the primers of the studied genes (*TNF-α*, *LITAF* and *BCL6*). To confirm the 100% efficiency of the designed primers, Nucleotide Blast (NCBI) and UCSC sites were used. According to Andrusiewicz et al. (23) study, one of the suitable housekeeping genes in the eutopic and ectopic endometrium is glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*); so this gene was used as housekeeping gene.

Sequences of the designed primers for *LITAF*, *TNF-α*,

BCL6 and *GAPDH* genes (Bioneer. Germany) are listed in Table 1.

Table 1: Primer pairs used in this study

Primers	Primer sequences (5´-3´)	Product size (bp)
LITAF	F: GCCTACCATTATCTTATCCGTC	84
	R: AATCTCAAAGCCAAGCCTG	
TNF-α	F: GGGCCTGTACCTCATCTA	212
	R: AGACCCCTCCCAGATAGATG	
BCL6	F: CCATTGTGAGAAGTGTAACCTG	172
	R: ACGAAAGCATCAACACTCC	
GAPDH	F: TGAGAAGTATGACAACAGCCTC	134
	R: TGATGGCATGGACTGTGGT	

Total RNA extraction and cDNA synthesis

Total RNA extraction was performed using TRIzol (Thermo Fisher Scientific, USA). To remove genomic contamination, the extracted RNA was treated by DNase I (Thermo Fisher Scientific, USA) before cDNA synthesis to remove genomic DNA. cDNA synthesis was done using the TaKara Kit (TaKara Bio, Japan, Cat # RR037A).

Quantitative reverse transcription polymerase chain reaction

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using the StepOnePlus 96-well Real-Time PCR system (Applied Biosystem, USA). For each reaction, 5 µl of SYBR Green (2X, Ampliqon, Denmark), 1 µl of the Forward Primer, 1 µl of the Reverse Primer, 11 µl water, 2 µl cDNA (concentration of 25 ng/µl) were combined to reach 20 µl total volume. Negative control (non-template water instead of cDNA) was performed in the all experiments. Data analysis was performed using the ΔCT (cycle threshold) method. ΔCT was calculated as the difference of the target gene Ct with the housekeeping gene Ct. The ΔΔCT was equal to the difference of ΔCT of the endometriosis sample with ΔCT of the control sample. Fold change was calculated by $2^{-\Delta\Delta CT}$.

Statistical analysis

Data were analyzed using One Way ANOVA (to compare gene expression between the three tissue groups) and SPSS software. Data were presented as mean ± standard error of the mean (SEM). A significant level was considered as $P<0.05$.

Ethical considerations

The study protocol was approved by Ethical Committee of Royan Institute for Reproductive Biomedicine, Iran. (IR.ACECR.ROYAN.REC.1398.007). Informed consent was obtained from the all participants to include them in the study.

Results

In this study, 20 women (10 women with endometriosis and 10 women without it) enrolled. The mean age of women with endometriosis and the control group are presented in Table 2. Gene expressions of *LITAF*, *BCL6*, *TNF-α* and *GAPDH* were detected in the endometrial samples (Fig.1). qRT-PCR analysis showed that *LITAF* gene expression was increased in the ectopic and eutopic tissues of women with endometriosis compared to the control endometrial samples. Although expression level of the *LITAF* gene in ectopic tissues was significantly higher than the controls (P=0.001) overexpression of this gene was not significant in the eutopic tissues of women with endometriosis compared to the controls (P=0.209). Additionally, *LITAF* gene expression was not significantly different in the ectopic and eutopic tissues of women with endometriosis (P=0.07, Fig.2A).

TNF-α gene expression wasincreased in the ectopic tissues of women with endometriosis compared to the control endometrium and eutopic tissues of women with endometriosis, but these increases were not significant (P=0.575 and P=0.308, respectively, Fig.2B).

Expression level of the *BCL6* gene in the ectopic tissues of women with endometriosis was significantly increased (P=0.000) compared to the both control and eutopic endometrial samples while its expression level was similar in the eutopic and control samples (Fig.2C).

Table 2: Number and mean age of the studied women

Tissue samples	Number of women	Menstrual cycle phase	No	Age (Y)
Endometriosis group	10	Proliferative phase:	8	29.9 ± 2.38
		Secretory phase:	2	
Control group endometrium	10	Proliferative phase:	9	31 ± 5.86
		Secretory phase:	1	

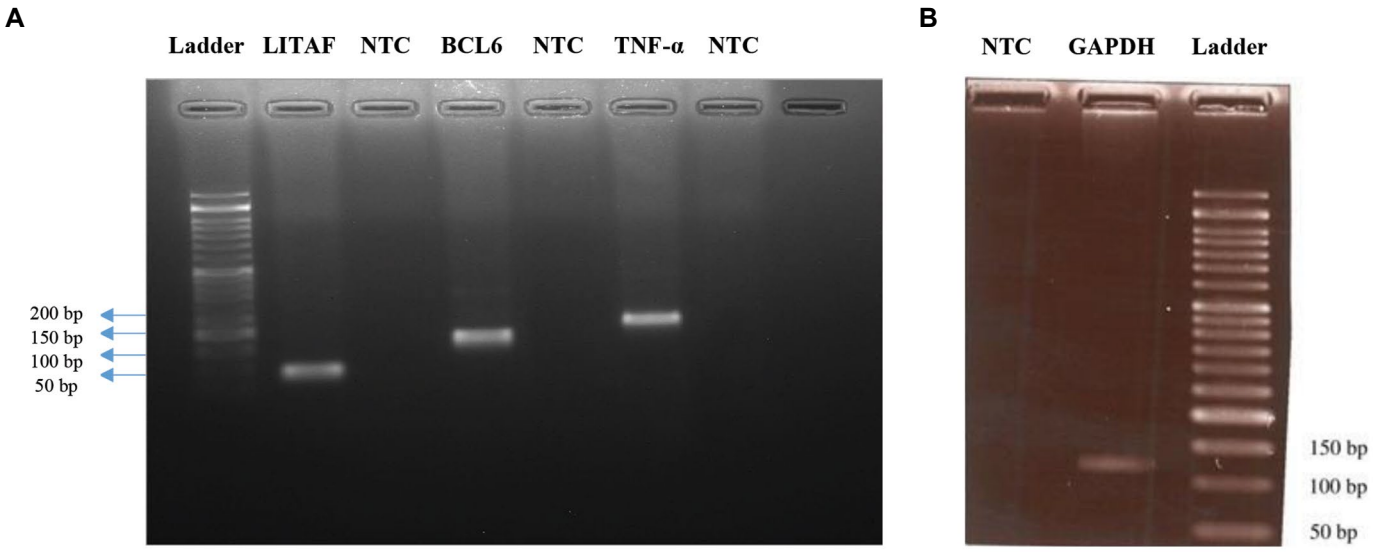

Fig.1: Evaluation of polymerase chain reaction (PCR) products on 1.5% agarose gel. **A.** Genes expressions of *LITAF*, *BCL6*, *TNF-α* and **B.** *GAPDH* in the eutopic endometrial samples. There was no amplified product in non-template control (NTC) samples.

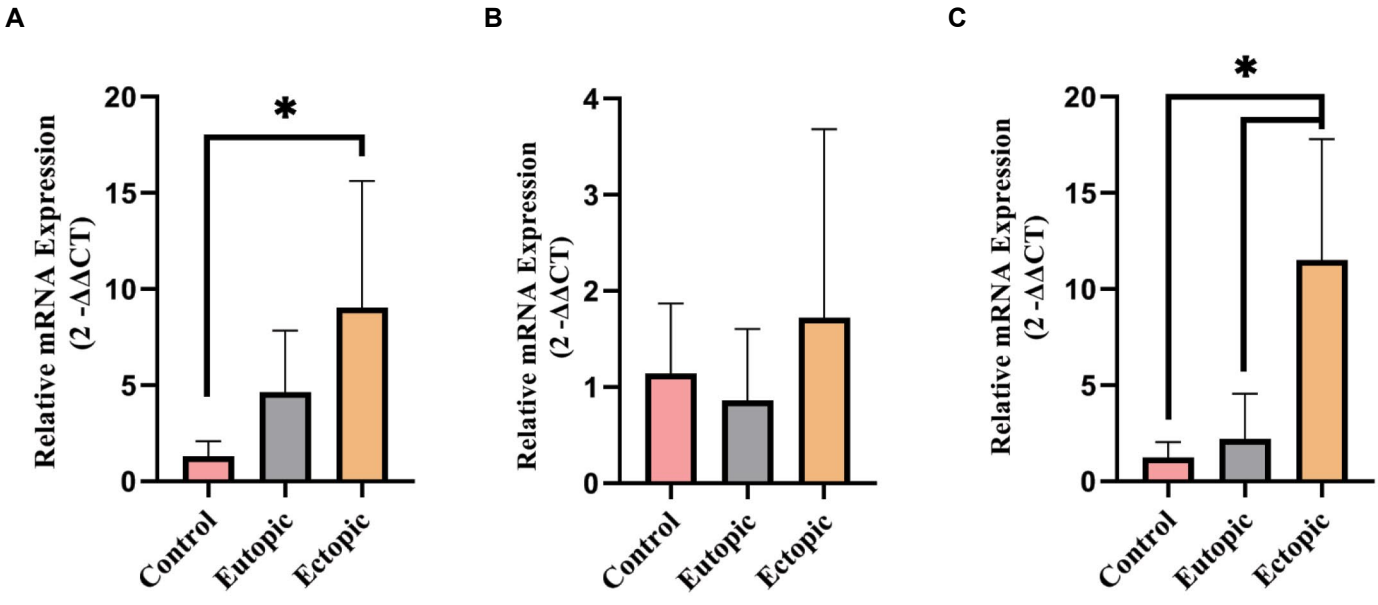

Fig.2: Expression profile of *LITAF, TNF-α,* and *BCL6* genes in three groups of the ectopic, eutopic and normal endometrial tissues. **A.** *LITAF*, **B.** *TNF-α*, and **C.** *BCL6* genes expression. The measures are provided as mean ± SEM (*; P<0.05).

Discussion

Endometriosis is defined as the presence of endometrial tissue in abnormal or ectopic locations. This disease is histologically defined as the presence of endometrial tissue or glands outside the uterine cavity (24). Improper placement of endometrial tissue outside the uterine cavity causes significant inflammation, with an increase in the number of active immune cells and widespread expression of inflammatory cytokines in endometrial tissue and peritoneal fluid of women with endometriosis (25). In the present study, gene expression levels of *TNF-α*, *LITAF* and *BCL6* were studied in the endometrial tissues of women with endometriosis in comparison with the control tissue samples and significant overexpression levels of *LITAF* and *BCL6* were detected in the ectopic lesions of women with endometriosis compared to the controls.

Immune cells and their secretions in the peritoneal fluid play an important role in the pathogenesis of endometriosis

(26). Macrophages secrete several cytokines, including IL-6 and TNF-α. Increased levels of IL-6 lead to the proliferation of endometrial cells (27). TNF-α as a cytokine of inflammatory response causes immune system disorder, localized pelvic adhesions and fibrosis in endometriosis leading to ectopic lesions (28). Increased TNF-α concentration in the peritoneal fluid leads to infertility in the affected women. This increase can affect sperm motility and interfere with the fertilization process (29).

In the present study, expression of *TNF-α* gene was increased in the ectopic endometrial tissues of women with endometriosis compared to the eutopic and control endometrial samples, but this increase was not significant. This overexpression pattern was consistent with Furucu et al. (30) and Richter et al. (13) studies. Furucu et al. (30) studied protein expression of TNF-α in 10 normal endometrial tissues and 24 endometriosis tissues by using immunohistochemistry method. They showed that TNF-α immunoreactivity was significantly increased in epithelial cells, stromal cells and macrophages in the endometriosis tissues compared to the control tissues. In this study, they emphasized that gene expression of *TNF-α* should be investigated. Richter et al. (13) showed that number of peritoneal macrophages and TNF-α secretion were significantly increased in peritoneal fluid of women with endometriosis. They studied 65 endometriosis women in comparison with 35 control women. In addition, Babaabasi et al. (31) showed that the -863 C/A polymorphism in the promoter region of the *TNF-α* gene may be involved in the incidence of endometriosis in Iranian women. TNF-α as one of main inflammatory cytokines can induce production of the IL-1, other growth factors (32) and TSLP (33). Our previous study on TSLP as an interleukin 7-like cytokine showed that gene expression level of *TSLP* was higher in the endometrial tissue of endometriosis rather than the control tissues (9). In addition, additive effect of 17β-estradiol (E2) and LPS, which stimulate peritoneal macrophages could lead to increased production of inflammatory cytokines such as, TNF-α and IL-6 which could contribute to the development of endometriosis by stimulating the immune system (34).

In addition, the present study showed that increased *LITAF* gene expression was significant in the ectopic lesions compared to the control endometrial tissues. This finding was in agreement with the previous report showing that expression levels of the *LITAF* gene and protein were increased in the eutopic tissues of women with endometriosis compared to the controls (17). They did not report any data with regard to ectopic tissues. In 2010, Khan et al. (35) showed that menstrual blood in women with endometriosis had higher level of contamination with *Escherichia coli* (E. coli) rather than women without endometriosis. They observed that TLR4 levels and endometrial cells growth were increased in response to LPS in women with endometriosis. Upon stimulation of monocytes or macrophages with LPS, LITAF transcription factor was transferred to the nucleus and encoded on the promoter of TNF-α inflammatory cytokine, which significantly increased transcription of this cytokine (16). Although in the present study, significant overexpression of *LITAF* was detected in the ectopic lesions of endometriosis group, overexpression of TNF-α was not significant. In addition, expression level of *TNF-α* gene was lower in comparison with *LITAF* gene.

In the current study, the results showed that *BCL6* gene expression in the ectopic tissues of women with endometriosis was significantly higher than the control group and eutopic tissues of women with endometriosis. In addition, expression level of the *BCL6* gene in the eutopic tissues of women with endometriosis was higher than the control group; but this increase was not significant. This increased expression level of *BCL6* in women with endometriosis was consistent with the results of the Evans-Hoeker et al. (19) and Yoo et al. (36) studies. Evans-Hoeker et al. (19) showed that expression level of the *BCL6* gene and protein were increased in the eutopic tissues women of endometriosis compared to the controls. Although they did not study ectopic lesions. Yoo et al. (36) showed that Kirsten rat sarcoma viral oncogene homolog (KRAS) was activated in the eutopic endometrial tissues of women with endometriosis, while activation of KRAS resulted in overexpression of Sirtuin 1 (*SIRT1*). They showed that BCL6 and SIRT1 were increased in the nucleus of endometrial cells of women with endometriosis leading to progesterone resistance and they were involved in the development of endometriosis.

Studies showed that IL-6 as an inflammatory cytokine was increased in the peritoneal fluid of women with endometriosis (37). IL-6 has been reported to increase the active form of STAT3 (phosphorylated protein) in the endometrium of women with endometriosis (38). On the other hand, pSTAT3 increased *BCL6* gene expression (39). This pathway could explain the overexpression of BCL6 in endometriosis.

In addition, it was shown that Silymarin (SMN), as an anti-inflammatory and anti-oxidant agent reduced ectopic lesions in mice by decreasing the glial cell-derived neurotrophic factor (GDNF), BCL2, and *BCL6* genes expression that can inhibit cell proliferation (40).

Shi et al. (21) showed the inverse correlation between genes and proteins expression of BCL6 and LITAF in B-cell non-Hodgkin lymphomas (B-NHL). LITAF suppressed the expression of the *BCL6* gene, and activated apoptosis via the mitochondrial pathway, which reduced cell proliferation. But in the present study, inverse correlation between *LITAF* and *BCL6* genes expression profile was not detected in the endometrial tissues of the studied groups. Our study showed the expression of *BCL6* and *LITAF* genes in the eutopic and ectopic tissues of women with endometriosis were increased in comparison with the control group.

Conclusion

Finally, it can be concluded that overexpression of *LITAF*, and *BCL6* genes in endometrial tissues (especially ectopic tissue) of women with endometriosis may be involved in the pathogenesis of this disease via altering inflammatory and cell cycling pathways. Increased expression of LITAF and then TNF-α may contribute to the spread of endometriosis disease by affecting the inflammatory pathway and apoptosis. On the other hand, increased expression of the *BCL6* gene may exacerbate the disease by affecting proliferation and growth of ectopic endometrial cells and interfering with progesterone signaling. Although these findings need to be confirmed in protein level with larger sample size.

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Authors' Contributions

A.S.V.; Data collection, Data analysis, Interpretation, and Drafting the manuscript. Sh.K.; Supported technical performance and Data analysis. F.R.; Gynecologist and the administrative supporter for collecting samples. M.Sh., E.A.; Designed and Supervised the research, and Took part in revising the manuscript. All authors studied and approved the final manuscript.

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