Original Article

# Evaluation of *Rap1GAP* and *EPAC1* Gene Expression in Endometriosis Disease

**Mehran Dehghanian[1,2], Ghafour Yarahmadi[1,2], Reyhaneh Sadat Sandoghsaz[2], Ali Khodadadian[1], Farimah Shamsi[3], Mohammad Yahya Vahidi Mehrjardi[4]**

[1]Department of Medical Genetics, Shahid Sadoughi University of Medical Sciences, Yazd, Iran, [2]Abortion Research Center, Yazd Reproductive Sciences Institute, Shahid Sadoughi University of Medical Sciences, Yazd, Iran, [3]Department of Biostatistics and Epidemiology, Center for Healthcare Data Modeling, School of Pulic Health, Shahid Sadoughi University of Medical Sciences, Yazd, Iran [4]Research Center for Food Hygiene and Safety, School of Public Health, Shahid Sadoughi University of Medical Sciences, Yazd, Iran

## Abstract

**Background:** Endometriosis is a female reproductive system disease in which the endometrial tissue is found in other women's organs. Various factors are effective in the development of endometriosis, and because of the interaction of genetics and environmental factors, this disease is a multi-factorial disease. MAPK/ERK and PI3K/Akt/mTOR pathways are activated by growth factors and steroid hormones and are known as two important pathways involved in the processes of growth, proliferation, and survival of endometriosis cells. Raps, monomeric GTPase of the Ras family, are able to activate these pathways independent of Ras. The goal of our study was to evaluate the expression level of *Rap1GAP* and *EPAC1* genes as two important RapGAPs (GTPase-activating proteins) and RapGEFs (guanine nucleotide exchange factors), respectively, in endometriosis tissues and normal endometrium tissues.

**Materials and Methods:** In this study, 15 samples of women without signs of endometriosis were taken as control samples. Fifteen ectopic and 15 eutopic samples were taken from women with endometriosis using laparoscopic surgery. The expression of *EPAC1* and *Rap1GAP* genes was investigated by the real-time polymerase chain reaction technique, and the results were analyzed by one-way ANOVA test.

**Results:** *EPAC1* upregulated significantly in ectopic tissues compared to eutopic and control tissues. *Rap1GAP* expression was lower in ectopic tissues compared to control and eutopic tissues.

**Conclusions:** Based on these results, it may be concluded that changes in the expression of the *Rap1GAP* and Epca1 genes may play a role in the pathways involved in the pathogenesis, displacement, and migration of endometriosis cells.

**Keywords:** Endometriosis, *EPAC1,* gene expression, *Rap1GAP*

**Address for correspondence:** Dr. Mohammad Yahya Vahidi Mehrjardi, Yazd Province, Yazd, Safaeih, BouAli, 89168-77391, Iran.
E-mail: mmvahidi@gmail.com
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## Introduction

Endometriosis is a benign complication caused by the growth of the endometrial tissue in other places, including the ovaries and fallopian tubes.[1] The real prevalence of endometriosis in the general population is not known precisely because a definitive diagnosis of the disease is possible only through invasive laparoscopic surgery; however, it is estimated that about 10% of women are probably affected by this disease.[2] Studies show that about 25–50% of infertile women have endometriosis, and 30–50% of women with this disease are infertile.[3]

The exact pathogenic mechanism of endometriosis is unknown; however, it is a multi-factorial disease in which genetic and environmental factors are involved. Numerous studies have examined genetic differences between affected and healthy

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individuals as well as between endometriosis and healthy tissues. They show that changes in some signaling pathways are involved in the development and progression of this disease, including the MAPK and PI3K/Akt pathways.[4-6]

*EPAC1* and *Rap1GAP* can alter signaling pathways associated with proliferation, migration, and apoptosis by affecting the activity of Rap and Ras proteins.[7,8] *EPAC1*, as a cAMP-activated GEF, can activate members of the *Rap* families and thereby can change the expression of MAPK pathway target genes.[9,10] *EPAC1* also activates the Akt pathway by Rap1 activation and then prevents apoptosis in cancer cells through activation of some anti-apoptotic proteins.[11]

*Rap1GAP* is known as a tumor suppressor in various cancers such as thyroid and breast cancers.[11,12] *Rap1GAP* affects the downstream pathways by activating the endogenous GTPase activity of Rap1.[13] Reduced *Rap1GAP* gene expression increases cell proliferation and decreases apoptosis through MAPK and Akt pathways in cancer cell lines.[12,14]

Accurate information on the expression of *EPAC1* and *Rap1GAP* genes in endometriosis is not available. This study aimed to investigate the expression of these genes in endometriosis tissues as well as normal endometrial tissues to find their possible effect on endometriosis.

## Materials and Methods

In this case-control study, we used 15 samples of ectopic and eutopic endometriosis tissues of women with endometriosis and 15 samples of normal endometrial tissues of women who had no signs of endometriosis or any lesion suggesting endometriosis referred to Shahid Sadoughi Hospital Yazd for reasons such as pelvic pain and fallopian tube blockage between April 2019 and March 2020. All women participating in this study were from the Iranian population living in Yazd. To maintain mRNA stability, the tissues were kept in RNA stabilizer solution (RNA later, Yekta Tajhiz Azma, Iran) at -80°C. The inclusion criteria in the two groups (women with endometriosis and without it) were as follows: the age range of the women in this study was 24–45 years. Samples were taken from all subjects in the study during the proliferative phase of the menstrual cycle; none of the women had taken any hormonal drugs in the 3 months before sampling, and no benign tumors or fibroids had been observed in any of the subjects. Also, endometrial cell changes such as fibroids and carcinoma and receiving hormonal medication in the last trimester were our exclusion criteria for both groups. All samples (cases and controls) were approved by the pathology laboratory for histological examination, and samples without contamination with other cell types were selected for examination.

### RNA extraction and cDNA synthesis

RNA total was extracted from 50 mg tissue samples by RNA X-plus Solution (CinnaGen, Iran), and the quality of the extracted RNA was measured using a nanodrop spectrophotometer. To investigate the expression level of genes in this study, cDNA was synthesized by reverse transcription reaction using a cDNA synthesis kit (Yekta Tajhiz Azma, Iran). For this purpose, 1 µg of total RNA was mixed with 1 µl random hexamer primer and brought to 13.5 µl by DEPC-treated water. After mixing, the material was incubated at 70°C for 5 minutes. In the next step, 4 µl 5x first-strand buffer, 1 µl dNTPs (10 mM each), 0.5 µl RNasin (40 U/µl), and 1 µl M-MLV reverse transcriptase were added to the mixture and incubated for 60 minutes at 37°C. In the last step, it was incubated for 5 minutes at 70°C and then kept at -20°C.

### qRT-PCR

In order to perform real-time quantitative polymerase chain reaction (PCR) reactions, the SYBR Green master mix (Yekta Tajhiz Azma, Iran) was used and the reactions were performed in a Rotor Gene-Q device (Qiagen, Germany) device. The thermal conditions of the reaction were 95°C for 15 min, followed by 40 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 30 s. Also, a final extension of 72°C for 5 min was performed. Our final mixing volume was 13 µl, including 1 µl cDNA, 0.5 µl forward primer, 0.5 µl reverse primer, and 6.25 µl of 2 × QPCR master mix. DNase-RNase-free water was added to reach a final volume of 13 µl. The *GAPDH* gene was selected as the internal control for *Rap1GAP* and *EPAC1* gene normalization. The primer's sequences are shown in Table 1.

### Statistical analysis

*EPAC1* and *Rap1GAP* gene expression levels in the three groups of ectopic, eutopic, and control were measured using the $2^{-\Delta\Delta Ct}$ method. To perform statistical analyses and compare the expression of *EPAC1* and *Rap1GAP* genes in ectopic and eutopic endometriosis and normal endometrial tissues, SPSS software (version 26) and one-way ANOVA with post-hoc Tukey's HSD test (for gene expression analysis) and T-test (for analysis of age) were used. $P$ value $< 0.05$ was considered statistically significant, and the plots were designed by GraphPad Prism 8.

## Results

The *Rap1GAP* expression level was evaluated by qPCR in endometriosis tissues as compared to the normal group. The analysis of data in eutopic and ectopic tissues of endometriosis patients as well as normal tissues indicated that *Rap1GAP* expression was not significantly different between eutopic endometriosis tissues and the normal endometrium and its expression was approximately equal in

**Table 1: Oligonucleotide primers**

| Gene | Primer sequence |
|---|---|
| *Rap1GAP* | Forward: 5`-GCACTTTCTCGGCAAGGAGCATTT-3` |
| | Reverse: 5`-TGACATCATGGTATGTCCGGCACT-3` |
| *EPAC1* | Forward: 5`-CATGAGGGAGATGATTTTGGA-3` |
| | Reverse: 5`-GCCTCCACATCCTTGATGATA-3` |
| *GAPDH* | Forward: 5`-CAAGAGCACAAGAGGAAGAGAGAG-3` |
| | Reverse: 5`-TCTACATGGCAACTGTGAGGAG-3` |

them (P-value = 0.958). According to the results, the *Rap1GAP* expression level in ectopic tissues was significantly reduced compared to control tissues (P-value = 0.003) and eutopic tissues (P-value = 0.001) [Figure 1a and Table 2].

*EPAC1* gene expression was also evaluated by qPCR in endometriosis tissues compared to the healthy group. Our findings demonstrated a significant increase in the expression level of the *EPAC1* gene in ectopic tissues of endometriosis patients as compared to the eutopic tissues and normal control tissues of healthy individuals (their *P* values were 0.000008 and 0.000096, respectively). There was not a significant difference in *EPAC1* gene expression in patients' eutopic tissues and the normal endometrium of healthy women (P-value = 0.714) [Figure 1b and Table 2].

There was no significant difference between the mean ages of the two groups participating in this study, patients with endometriosis and those without endometriosis (P-value = 0.3852) [Table 3].

## Discussion

Rap1, as a small GTPase, is one of the *Ras* family genes. Rap1 binds to both GDP and GTP, and the change between the two states describes it as a molecular switch.[15,16] Rap1 is involved in various signaling pathways, including the MAPK/ERK and PI3K/AKT signaling pathways, and by affecting these signaling pathways, it can change the rate of cell proliferation, migration, and cell invasion.[8,17] The regulation of RAP1 activity in the cells is performed by two classes of proteins. GEFs (guanine nucleotide exchange factors), such as *EPAC1* and PDZ-GEFs, help to change the bound nucleotide to Rap1 easier, and this protein can re-bind to more abundant GTP in the cells and then increase its activation. GAP (GTPase-activating proteins), such as *Rap1Gap* and *SPA-1*, increases the natural GTPase activity of Rap1 and then decreases its activation.[18] Many studies have shown that changes in the activity of the MAPK and PI3K/AKT signaling pathways are associated with endometriosis, and changes in Rap1 and its regulators such as GAPs and GEFs may have a role in the pathogenesis of endometriosis.[19]

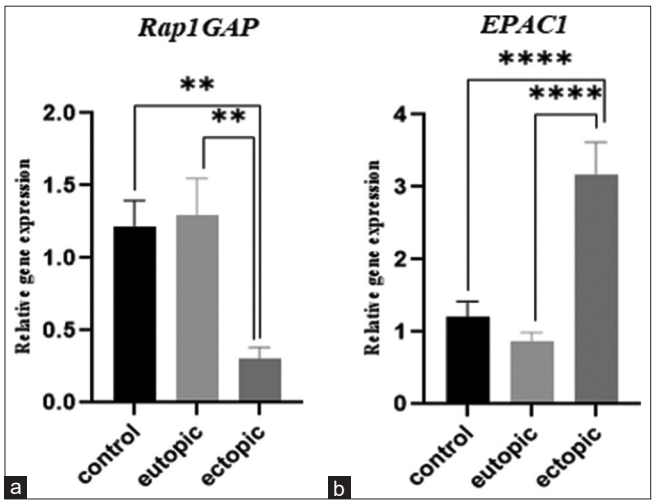


**Figure 1:** Comparison of *EPAC1* and *Rap1GAP* gene expression with *GAPDH* gene expression in the ectopic, eutopic, and control tissues. (a) The ratio of *Rap1GAP* to *GAPDH* and (b) the ratio of gene expression of *EPAC1* to *GAPDH.* One-way ANOVA with post-hoc Tukey's HSD test was used

*Rap1GAP* is known in various cancers as a tumor suppressor, and its expression has been reduced in thyroid and gastric cancers.[20,21] Decreased *Rap1GAP* expression can alter the expression of the MAPK and PI3K/Akt pathways' target genes, resulting in increased cell growth and migration and proliferation in human umbilical vein endothelial cells (HUVECs).[22] In 2003, Kao LC *et al*.[23] examined the expression profile of genes in endometriosis tissues of patients by using the micro-array method and concluded that the expression of the *Rap1GAP* gene in endometriosis tissues is reduced compared to normal endometrial tissues. By examining the expression of the *Rap1GAP* mRNA level in ectopic and eutopic tissues and comparing them with the expression of this gene in normal endometrial tissues, it seems that decreased expression of this gene is observed in endometriosis ectopic tissues, which may be related to the role of this gene in regulating the pathways involved in endometriosis cell migration and displacement in this disease.

*EPAC1*, as a GEF enabled by cAMP, can replace GDP with GTP in Raps and activate them. *EPAC1* is activated by growth factors through GPCRs (G-protein-coupled receptors).[24] Studies have shown that increased expression of this gene in various cancers such as ovarian and breast can increase proliferation, migration, and cell invasion.[7,25] The study of prostate cancer cell lines has shown that increasing *EPAC1* expression through activating MAPK and Akt signaling pathways and ultimately activating mTOR increases cell survival and cell proliferation.[9] It has been shown that knockdown of *EPAC1* by siRNA in ovarian cancer cell lines can reduce the activity of the PI3K/Akt signaling pathway so that by decreasing the expression of *EPAC1*, the amount of p-Akt is reduced. Decreasing the amount of p-Akt in ovarian

**Table 2: Means and standard error of the mean (SEM) of *Rap1GAP* and *EPAC1* fold change (FC)**

| Groups | Mean±SEM | |
|---|---|---|
| | *Rap1GAP* | *EPAC1* |
| Control | 1.220±0.1757 | 1.207±0.2145 |
| Eutopic | 1.293±0.2568 | 0.8764±0.1141 |
| Ectopic | 0.3050±0.07725 | 3.165±0.4548 |

**Table 3: Demographic data of the participants**

| Groups | Patients* | Age (yr)** |
|---|---|---|
| Case (Ectopic and Eutopic) | 15 | 33.53±1.2 |
| Control | 15 | 35.27±1.5 |

* Data are presented as total numbers of each variable. **Data are presented as mean±SEM

cancer cell lines reduces the expression of target genes in this pathway, including cyclin D1 and CDK4, and causes the cell to remain in the G1 phase of the cell cycle. These results showed that increasing the expression of the *EPAC1* gene in ovarian cancer cells increases cell proliferation and survival and reduces cell apoptosis.[25]

Because there was no study on the expression of the *EPAC1* gene in endometriosis tissues and according to previous studies that have pointed to the role of MAPK and PI3K/Akt pathways in the incidence of endometriosis, in this study, we evaluated the expression level of *EPAC1* mRNA in the endometriosis. In this study, it was found that the expression of the *EPAC1* gene in ectopic tissues has increased compared to eutopic tissues and the normal endometrium. These results may indicate that increased expression of the *EPAC1* gene could play a role in endometriosis progression, migration, and displacement of endometriosis cells during endometriosis.

## Conclusion

The results of this study showed that the expression of the *Rap1GAP* gene, as one of the factors that inhibit the activity of MAPK and Akt signaling pathways through Raps proteins, did not show any significant difference in normal endometrial and eutopic tissues; however, its expression was down-regulated in ectopic tissues. On the other hand, the *EPAC1* gene, which is one of the activators of Raps proteins, shows increased expression only in ectopic tissues of patients with endometriosis compared to eutopic tissues of patients and the normal endometrium. These results suggest that increased *EPAC1* expression and decreased expression of *Rap1GAP* in ectopic tissues may activate pathways involved in the progression and migration of endometriosis cells to other organs. Given that various factors can play a role in the activity of MAPK and Akt pathways involved in the development of endometriosis, it is suggested that other genes involved in these pathways and epigenetic factors affecting the activity of these pathways be investigated in future studies.

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### Ethics approval and consent to participate

This study and the whole process of holding it has been done under the supervision of the ethics committee of Shahid Sadoughi University of Medical Science, Yazd, Iran, and informed consent has been obtained from all patients and participants in this study.

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### Conflicts of interest

There are no conflicts of interest.

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