



Evaluation of NF1 and RASA1 gene expression in endometriosis

Ghafour Yarahmadi^{a,d}, Mehran Dehghanian^{a,d}, Reyhaneh Sadat Sandoghsaz^d,
Mohamadreza Savaee^c, Farimah Shamsi^d, Mohammad Yahya Vahidi Mehrjardi^{b,d,*}¹

^a Department of Medical Genetics, Shahid Sadoughi University of Medical Sciences, Yazd, Iran

^b Research Center for Food Hygiene and Safety, School of Public Health, Shahid Sadoughi University of Medical Sciences, Yazd, Iran

^c Department of Clinical Biochemistry, School of Medicine, Shahid Sadoughi University of Medical Sciences and Health Services, Yazd, Iran

^d Abortion Research Center, Yazd Reproductive Sciences Institute, Shahid Sadoughi University of Medical Sciences, Yazd, Iran

ARTICLE INFO

Keywords:

Endometriosis
RASA1
NF1
PI3K / Akt
MAPK

ABSTRACT

Background: Endometriosis affecting 6–10% of women of reproductive ages around the globe. Important pathways, including the MAPK and PI3K / Akt pathways, have been identified in the disease. The NF1 and RASA1 genes inactivate Ras by their own GTPase activity and controlled the high activity of these pathways.

Objective: In this study, we measured NF1 and RASA1 gene expression in the endometrial tissues of patients (eutopic and ectopic tissues) compared to the control samples.

Materials and methods: In our study, tissue samples were collected from 15 patients with endometriosis and 15 healthy women. We used quantitative polymerase chain reaction (qRT-PCR) to measure the NF1 and RASA1 gene expression levels in these samples.

Results: We observed a significant decrease in the expression level of the NF1 gene in both eutopic and ectopic samples of endometriosis patients compared to control samples, while the expression of the RASA1 gene was significantly reduced only in ectopic tissues.

Introduction

Endometriosis, as an estrogen-dependent disease, is one of the most common abnormalities among women. In this disease endometrial tissues, including the mesenchyme and stroma, grow outside the uterine cavity [1]. It affects approximately 10% of women of childbearing age and 20–50% of women with infertility problems [1–3]. These tissues grow in the ovaries, pelvis, peritoneal cavity, vaginal, anus wall and in rare cases are found in the diaphragm, pericardium and pleura. Some symptoms are common in endometriosis patients such as painful menstruation, chronic pelvic pain, abnormal bleeding from the uterus and reduced fertility and infertility [4–6]. Although the exact cause of this disease is not yet clear [7], many studies have shown that endometriosis is a multifactorial disease and several factors are involved in its pathogenesis such as signaling pathways, hormonal activity, immune system problems, environmental factors and genetic factors [8]. The role of genetic factors including aberrant expression of genes and important signaling pathways has been demonstrated [9].

Some studies have shown that the MAPK (mitogen-activated protein

kinase) pathway plays an important role as extracellular and intracellular signaling in endometrial cells, then its abnormal activation can involve in the pathogenesis of endometriosis [10,11]. One of the main pathways downstream of many EGF (epidermal growth factor) receptors is the RAS-mediated signal, which activates the MAPK pathway [12]. Ras proteins that are constantly rotating between active Ras-GTP and inactive Ras-GDP are located mainly on the inner surface of the plasma membrane [13]. Several pathways, including PI3K / AKT and MAPK, which play an important role in cell survival and growth, can be interconnected and activated by Ras linked to GTP [14]. RASA1 and NF1 proteins, which are RasGAPs (Ras-GTPase Activating Proteins), terminate the active Ras state by hydrolyzing GTP to GDP, thus negatively regulating the Ras pathway [15]. Accordingly, the development of various mutations and disruption of the expression of this tumor suppressor RasGAPs can lead to the formation of various types of cancers as well as endometriosis [16,17].

In this study, our aim was to investigate the expression of NF1 and RASA1 genes in patients with endometriosis (both ectopic and eutopic samples) compared with healthy individuals.

* Corresponding author at: Research Center for Food Hygiene and Safety, School of Public Health, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.
E-mail address: mmvahidi@ssu.ac.ir (M.Y. Vahidi Mehrjardi).

¹ (ORCID ID 0000-0003-0535-1590)

Table 1
Oligonucleotide primers.

Gene	Primer sequence
NF1	Forward: 5'-CGAATGGCACCGAGTCTT AC-3' Reverse: 5'-GACCAGTTGGACGAGCCC - 3'
RASA1	Forward: 5'-CCAACGCCAAACAATCAG - 3' Reverse: 5'-ATTTCTTGCCATCCACT - 3'
GAPDH	Forward: 5'-CAAGAGCACAAAGAGGAAGAGAGAG-3' Reverse: 5'-TCTACATGGCAACTGTGAGGAG-3'

Materials and methods

According to the ethical rules, the general consent of all patients was obtained consciously and freely. This study has also been approved by the Ethics Committee of Yazd Institute of Reproductive Sciences, Shahid Sadoughi University of Medical Sciences, Yazd, Iran. From September 2020 to February 2021, 45 samples were collected from women, who referred to Madar and Shahid Sadoughi hospitals. Normal endometrium (15 samples) and endometriosis tissues (15 ectopic and 15 eutopic) were collected during surgery. Normal endometrium samples were collected from women who were hospitalized for other reasons such as fallopian tube closure and pelvic pain who did not have endometriosis. These samples were placed in microtubes containing RNA later solution (CinnaGen, Iran) and stored at -80°C . Inclusion criteria were that all women who underwent surgery were between 20 and 45 years old and did not receive any hormone therapy until 6 months before surgery, as well as exclusion criteria, included the presence of benign tumors polyps (fibroids), hyperplasia, carcinoma, and human papillomavirus infection.

Quantitative real-time PCR and RNA isolation

We extracted total RNA from the tissues of patients and healthy

individuals using RNA xPlus Solution (Cinna Gen, Iran). We determined the RNA concentration and purity by using OD 260/280 and a spectrophotometer (NanoDrop, Thermo Fisher). cDNA (complementary DNA) of the NF1 and RASA1 was synthesized by using a cDNA synthesis kit (Yekta Tajhiz Azma, Iran) according to its instructions. Real-time PCR was performed separately for NF1 and RASA1 and run in a Rotor GeneQ device (Qiagen, Germany) to check their expression. We used GAPDH for the reference gene for NF1 and RASA1 (Table 1).

Statistical analysis

Statistical analysis was done by one-way ANOVA using GraphPad Prism software (Version 7.3). We considered $p < 0.05$ for a significant difference. The figures in this article are also drawn by Prism7.3 software.

Result

Real-time PCR data analysis showed that NF1 gene expression was significantly different between patients and control samples. NF1 gene, in the ectopic group compared to the control group, showed a significant decrease ($p = 0.0024$), while, its expression did not change much compared to the eutopic group ($p = 0.8773$). The expression level of the NF1 gene in the eutopic samples also had a significant decrease compared to the control samples ($p = 0.0093$) (Fig. 1A).

RASA1 expression in the ectopic group showed a significant decrease compared to the control group ($p = 0.0346$), while, its expression did not change significantly compared to the eutopic group ($p = 0.3470$). The expression of this gene in the eutopic group there was no significant difference compared to the control ($p = 0.4708$) (Fig. 1B).

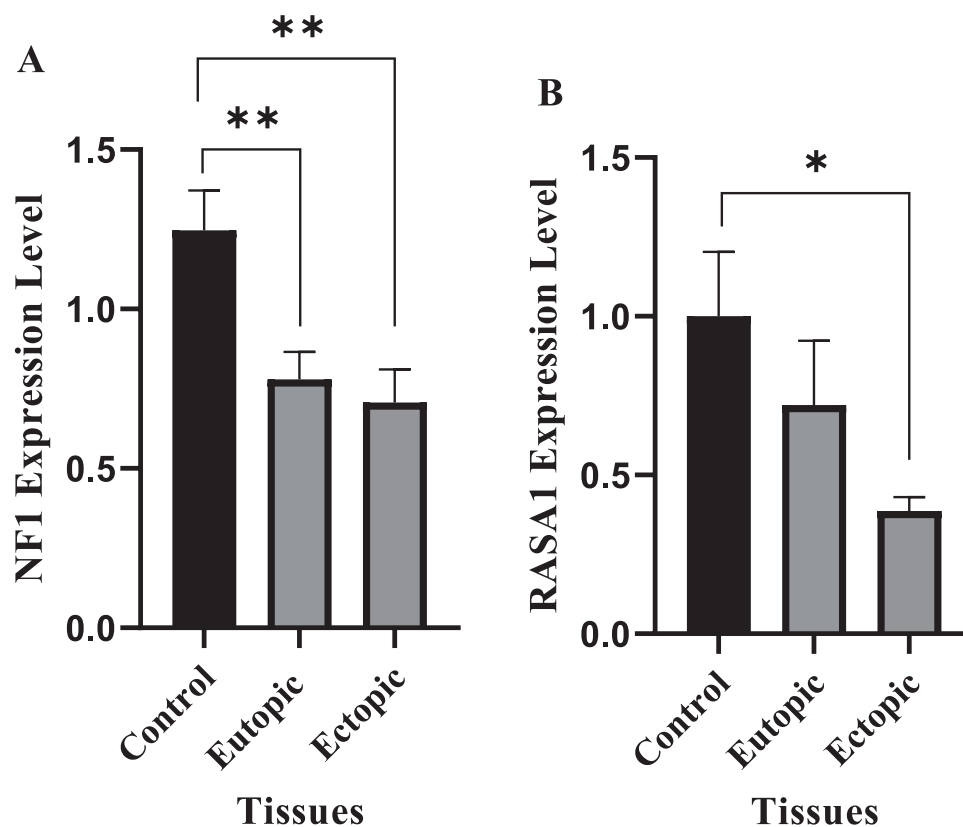


Fig. 1. comparison of NF1 and RASA1 gene expression with GAPDH gene expression in three ectopic, eutopic and control tissues. The ratio of NF1 to GAPDH (A) and the ratio of gene expression of RASA1 to GAPDH (B).

Discussion

Endometriosis originates from the uterus, which is a reproductive tissue of women [18]. Endometriosis tissues located at other parts of the body, especially in the ovaries [19]. Some processes in endometriosis such as transmission to other tissues, growth and proliferation is similar to female reproductive cancer. In fact, it is a precancerous condition and several studies have been performed to investigate its molecular processes [20,21]. The MAPK signaling pathway is very important in regulating cellular processes such as survival, differentiation, proliferation, apoptosis, inflammation and innate immune processes [22]. Therefore, the impaired activity of any of the components of this pathway can cause metastasis, invasion and formation of various tumors [23]. In addition, PI3K / Akt signaling, which is involved in processes such as angiogenesis, cell proliferation, and survival, has been shown to be an important pathway in the development of endometriosis [24]. Ras in its active state (connected to GTP) activates the MAPK and PI3K / Akt paths [25]. NF1 and RASA1 genes are tumor suppressors that inhibit RAS activity by acting as GAP [26]. As a result, the activity of the MAPK and PI3K / Akt pathways is either reduced or inhibited [26].

The RASA1 gene plays an important role in controlling the angiogenesis process [27]. Studies have shown that reducing the function of the RASA1 gene interferes with the regulation of the angiogenesis process and causes various cancers, such as breast cancer [16,28]. It has been shown that increasing RASA1 levels by suppressing miR-145 can reduce the progression of ovarian cancer [29]. In a study by Zheng et al. on hepatocellular carcinoma, it was found that in hypoxic condition, decreased RASA1 expression increased angiogenesis [30]. In the development of advanced endometriosis, increased angiogenesis can have a significant effect and cause the disease to spread to different organs [31]. The results of this study showed that RASA1 expression level in ectopic tissues was reduced compared to control and eutopic tissues. Based on this, it can be concluded that decreased expression of the RASA1 gene can lead to the formation of endometriosis, especially its advanced stage.

NF1 (encoding the neurofibromin protein 1) is the major mutated gene in the germline of patients with neurofibromatosis type 1, which disrupts the regulation of the RAS / MAPK pathway [32]. Studies have shown that people with the disease with NF1 loss-of-function mutation, have a 5 times higher risk of breast cancer than the general population [33]. Jiang et al.'s study on ovarian cancer showed that reducing NF1 gene expression as a tumor suppressor stopped apoptosis and increased the growth and proliferation of cancer cells, and in a separate study, the NF1 gene mutation was found only in endometriosis-related ovarian cancer [34,35]. Our study showed that NF1 gene expression was decreased in ectopic and eutopic samples of patients compared with control samples of healthy individuals. According to this result, we can conclude that the NF1 gene plays an important suppressive role both in the early stages of endometriosis and in its advanced stages, and reducing its expression stops apoptosis, increases the proliferation and invasion of endometriosis cells.

Since no study was performed on the expression of RASA1 and NF1 genes in endometriosis and due to the regulatory role of these two genes in the MAPK and PI3K / Akt pathways, in this study we examined the level of expression of these genes in endometriosis tissues (ectopic and eutopic) and normal endometrial tissue.

Conclusion

The results of this study showed that NF1 and RASA1 genes play a role in tumor suppression in endometriosis and can control activated signaling pathways (MAPK and PI3K / Akt) in this disease through negative regulation. Decreased expression of NF1 and RASA1 genes may be a new biomarker for the prognosis of the disease if patients' clinical outcomes and expression characteristics of NF1 and RASA1 genes are determined.

Acknowledgments

We thank the study co-workers in Yazd Institute of Reproductive Sciences for their help and selfless contributions to this project.

Conflict of interest

The authors pronounced that they have no dispute interest.

References

- Osiński M, Wirstlein P, Wender-Ożegowska E, Mikołajczyk M, Jagodziński PP, Szczepańska M. HSD3B2, HSD17B1, HSD17B2, ESR1, ESR2 and AR expression in infertile women with endometriosis. *Ginekol. Polska* 2018;89(3):125–34.
- Haikal ME, Wessels JM, Leyland NA, Agarwal SK, Foster WG. MicroRNA expression pattern differs depending on endometriosis lesion type. *Biol. Reprod.* 2018;98(5):623–33.
- Petracco R, Dias ACDO, Taylor HS, Petracco Á, Badalotti M, Michelson JDR, et al. Evaluation of miR-135a/b expression in endometriosis lesions. *Biomed. Rep.* 2019; 11(4):181–7.
- Miyashita M, Koga K, Takeuchi A, Makabe T, Taguchi A, Urata Y, et al. Expression of nerve injury-induced protein1 (Nin1) in endometriosis. *Reprod. Sci.* 2018;26 (8):1105–10.
- Bjorkman S, Taylor HS. MicroRNAs in endometriosis: biological function and emerging biomarker candidates. *Biol. Reprod.* 2019;101(6):1167–78.
- Hu Z, Mamillapalli R, Taylor HS. Increased circulating miR-370-3p regulates steroidogenic factor 1 in endometriosis. *Am. J. Physiol. Endocrinol. Metabol.* 2019; 316(3):E373–82.
- Wuyung PE, Rahadiati FB, Hartono Tjahjadi SS, Kusmardi K, Kodariah R, Wiweko B. Histopathology and ARID1A expression in endometriosis-associated ovarian carcinoma (EAOC) carcinogenesis model with endometrial autoimplantation and DMBA induction. *Asian Pac. J. Cancer Prevent. APJCP* 2021; 22(2):553.
- Malvezzi H, Marengo EB, Podgaec S, de Azevedo Piccinato C. Endometriosis: current challenges in modeling a multifactorial disease of unknown etiology. *J. Transl. Med.* 2020;18(1):1–21.
- Bouaziz J, Mashach R, Cohen S, Kedem A, Baron A, Zajicek M, et al. How artificial intelligence can improve our understanding of the genes associated with endometriosis: natural language processing of the PubMed Database. *BioMed Res. Int.* 2018;2018.
- Cheng AM, Saxton TM, Sakai R, Kulkarni S, Mbamalu G, Vogel W, et al. Mammalian Grb2 regulates multiple steps in embryonic development and malignant transformation. *Cell.* 1998;95(6):793–803.
- Kim EK, Choi E-J. Compromised MAPK signaling in human diseases: an update. *Arch. Toxicol.* 2015;89(6):867–82.
- Watanabe T, Shinohara N, Moriya K, Sazawa A, Kobayashi Y, Ogiso Y, et al. Significance of the Grb2 and son of sevenless (Sos) proteins in human bladder cancer cell lines. *IUBMB Life* 2000;49(4):317–20.
- Nichols RJ, Haderk F, Stahlhut C, Schulze CJ, Hemmati G, Wildes D, et al. Efficacy of SHP2 phosphatase inhibition in cancers with nucleotide-cycling oncogenic RAS, RAS-GTP dependent oncogenic BRAF and NF1 loss. *bioRxiv* 2017:188730.
- Erdogan S, Turkecul K, Dibirdik I, Doganlar O, Doganlar ZB, Bilir A, et al. Midkine downregulation increases the efficacy of quercetin on prostate cancer stem cell survival and migration through PI3K/AKT and MAPK/ERK pathway. *Biomed. Pharmacother.* 2018;107:793–805.
- Bellazzo A, Collavin L. Cutting the brakes on ras—cytoplasmic GAPs as targets of inactivation in cancer. *Cancers* 2020;12(10):3066.
- Zhang Y, Li Y, Wang Q, Su B, Xu H, Sun Y, et al. Role of RASA1 in cancer: a review and update. *Oncol. Rep.* 2020.
- Brosseau J-P, Liao C-P, Wang Y, Ramani V, Vandergriff T, Lee M, et al. NF1 heterozygosity fosters de novo tumorigenesis but impairs malignant transformation. *Nat. Commun.* 2018;9(1):1–11.
- Freytag D, Mettler L, Maass N, Günther V, Alkatout I. Uterine anomalies and endometriosis. *Minerva Med.* 2019;111(1):33–49.
- Murakami K, Kotani Y, Nakai H, Matsumura N. Endometriosis-associated ovarian cancer: the origin and targeted therapy. *Cancers* 2020;12(6):1676.
- McCluggage WG. Endometriosis-related pathology: a discussion of selected uncommon benign, premalignant and malignant lesions. *Histopathology* 2020;76 (1):76–92.
- Melin A, Sparen P, Persson I, Bergqvist A. Endometriosis and the risk of cancer with special emphasis on ovarian cancer. *Human Reprod.* 2006;21(5):1237–42.
- Guo YJ, Pan WW, Liu SB, Shen ZF, Xu Y, Hu LL. ERK/MAPK signalling pathway and tumorigenesis. *Exp. Ther. Med.* 2020;19(3):1997–2007.
- Rezatabar S, Karimian A, Rameshkhnia V, Parsian H, Majidinia M, Kopi TA, et al. RAS/MAPK signaling functions in oxidative stress, DNA damage response and cancer progression. *J. Cell. Physiol.* 2019;234(9):14951–65.
- Barra F, Desideri LF, Ferrero S. Inhibition of PI3K/AKT/mTOR pathway for the treatment of endometriosis. *Br. J. Pharmacol.* 2018;175(17):3626.
- Butler DE, Marlein C, Walker HF, Frame FM, Mann VM, Simms MS, et al. Inhibition of the PI3K/AKT/mTOR pathway activates autophagy and compensatory Ras/Raf/MEK/ERK signalling in prostate cancer. *Oncotarget* 2017;8(34):56698.

- [26] Li L, Fan Y, Huang X, Luo J, Zhong L, Shu X-s, et al. Tumor suppression of Ras GTPase-activating protein RASA5 through antagonizing Ras signaling perturbation in carcinomas. *Iscience* 2019;21:1–18.
- [27] Xiao W, Zheng S, Zou Y, Yang A, Xie X, Tang H, et al. CircAHNAK1 inhibits proliferation and metastasis of triple-negative breast cancer by modulating miR-421 and RASA1. *Aging* 2019;11(24):12043.
- [28] Cao Y, Chu C, Li X, Gu S, Zou Q, Jin Y. RNA-binding protein QKI suppresses breast cancer via RASA1/MAPK signaling pathway. *Ann. Transl. Med.* 2021;9(2).
- [29] Hu J, Wang L, Chen J, Gao H, Zhao W, Huang Y, et al. The circular RNA circ-ITCH suppresses ovarian carcinoma progression through targeting miR-145/RASA1 signaling. *Biochem. Biophys. Res. Commun.* 2018;505(1):222–8.
- [30] Du C, Weng X, Hu W, Lv Z, Xiao H, Ding C, et al. Hypoxia-inducible MiR-182 promotes angiogenesis by targeting RASA1 in hepatocellular carcinoma. *J. Exp. Clin. Cancer Res.* 2015;34(1):1–9.
- [31] Samimi M, Pourhanifeh MH, Mehdizadehkashi A, Eftekhari T, Asemi Z. The role of inflammation, oxidative stress, angiogenesis, and apoptosis in the pathophysiology of endometriosis: basic science and new insights based on gene expression. *J. Cell. Physiol.* 2019;234(11):19384–92.
- [32] Osum SH, Coutts AW, Duerre DJ, Tschida BR, Kirstein MN, Fisher J, et al. Selumetinib normalizes Ras/MAPK signaling in clinically relevant neurofibromatosis type 1 minipig tissues in vivo. *Neuro Oncol. Adv.* 2021;3(1):vdab020.
- [33] Kiuru M, Busam KJ. The NF1 gene in tumor syndromes and melanoma. *Lab. Invest.* 2017;97(2):146–57.
- [34] Su J, Ruan S, Dai S, Mi J, Chen W, Jiang S. NF1 regulates apoptosis in ovarian cancer cells by targeting MCL1 via miR-142–5p. *Pharmacogenomics.* 2019;20(03):155–65.
- [35] Guo SW. Cancer driver mutations in endometriosis: variations on the major theme of fibrogenesis. *Reprod. Med. Biol.* 2018;17(4):369–97.