Altered VEGF, Bcl-2 and IDH1 expression in patients with adenomyosis

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Abstract

Purpose Adenomyosis is a benign uterine disease resulting from the myometrial invasion of the endometrial gland and stroma. In the current study, angiogenesis, apoptosis and energy metabolism were investigated in adenomyosis.

Methods A retrospective study was performed using paraffin archival tissues. Three groups were included in the study: Group I and Group II; ectopic and eutopic endometrial tissues of patients with adenomyosis, respectively, and Control Group; endometrial tissue of individuals without adenomyosis. Vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), intercellular adhesion molecule 1 (ICAM-1) and hypoxia-inducible factor 1 alpha (HIF-1A) levels were evaluated as angiogenic markers. Bcl-2, caspase-9 and caspase-3 levels were investigated as apoptotic indicators, and isocitrate dehydrogenase 1 (IDH1), succinate dehydrogenase complex subunit C (SDHC) and fumarate hydratase (FH) levels were also examined as energy metabolism markers. Gene expression levels of all parameters were determined by RT-PCR.

Result VEGF expression levels were found to be increased in Group I according to the control group and Group II. Bcl-2 expression levels were found to be increased in the Group I compared to the Group II. It was determined that expression levels of IDH1 were decreased in the Group I and Group II compared to the Control Group. There was no significant difference in the other examined parameters. Although we did not find a significant difference in HIF-1A levels between the groups, we found a positive correlation between VEGF and HIF-1A in the Group I.

Conclusion These results point out that VEGF, HIF-1A, Bcl-2 and IDH1 may be associated with the etiology of adenomyosis.

Keywords Adenomyosis · VEGF · Bcl-2 · IDH1 · HIF-1A

Introduction

Adenomyosis is a benign uterine disease characterized by the presence of endometrial glands and stroma in the myometrium [1, 2]. The most common symptoms are abnormal uterine bleeding, menorrhagia, dysmenorrhea and pelvic pain [1]. Adenomyosis may also be asymptomatic in some individuals. Although it is a common disease, its etiology and pathogenesis remain unclear [3, 4]. Adenomyosis is usually associated with other benign disorders such as endometriosis, leiomyomas, endometrial hyperplasia and endometrial polyps [5].

Most cases of adenomyosis are detected in women at the end of the reproductive age (40–50 years) [6]. The patients are diagnosed with adenomyosis by histopathological analysis after hysterectomy; therefore, the exact incidence of adenomyosis is still unclear [7]. The incidence of adenomyosis in hysterectomy specimens is 20–30%, and it is observed more frequently in multiparous patients [8]. The molecular mechanism of adenomyosis is uncertain [2, 9]. Increased estrogen exposure [10], the metaplastic transformation of embryological pluripotent mullerian residues [5], invagination of the basal endometrium to the myometrium and intramyometrial spreading [11] and tissue damages such as having undergone uterine surgery have been suggested to be involved in the etiology of adenomyosis [12]. Treatment options for adenomyosis include both medication and

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surgical treatment. However, the standard treatment method used today is hysterectomy [2, 13].

Histological examinations showed that the endometrium of women with adenomyosis had different vascularization than healthy endometrium [14]. Angiogenic factors suggested to be responsible for abnormal endometrial vascularization in adenomyosis are vascular endothelial growth factor (VEGF) and hypoxia-inducible factor-1 alpha (HIF-1A) [15]. The high activity of these molecules leads to abnormal development in both the proliferative and secretory phases. It has been offered that VEGF is an important factor responsible for abnormal endometrial vascularization in adenomyosis [15, 16]. It is thought that anti-apoptotic processes due to Bcl-2 facilitate the invasion and intramyometrial spread of the endometrium to the myometrium in adenomyosis [11]. Nevertheless, the mechanism of apoptosis in adenomyosis and therefore the role of molecules such as Bcl-2 and caspases have not been clear. There are few studies on this issue and especially different findings related to Bcl-2 [17, 18]. The ideas about the role of apoptosis in this disease are insufficient. However, the general opinion is that apoptosis is suppressed in adenomyosis. The effect of cellular energy metabolism on the development of adenomyosis is not known. There is no other study in which Krebs cycle enzymes were investigated in adenomyosis.

The molecular mechanism of adenomyosis development has not yet been fully elucidated. Sex steroid hormone abnormalities, the presence of inflammation, altered cell proliferation and angiogenesis related to hypervascularization are recognized as possible key pathogenic mechanisms in adenomyosis. We aimed to contribute to the elucidation of this mechanism by examining the state of angiogenesis, apoptosis and energy metabolism in adenomyosis.

Materials and methods

Collection of tissue samples

A total of 90 paraffin-embedded archival tissues and three groups were included in as follows: Group I; ectopic endometrial tissues of adenomyosis patients ($n = 35$), Group II; eutopic endometrial tissues (endometrial tissues that normally located in the inner layer of the uterus) of adenomyosis patients ($n = 35$), Control Group; endometrial tissues of individuals without adenomyosis ($n = 20$). These tissues were surgically collected from 35 patients clinically and histopathologically diagnosed with adenomyosis and from 20 individuals without adenomyosis as a control group between June 2014 and May 2017 in the Department of Obstetrics and Gynecology and Department of Pathology, Hospital of Mersin University (Mersin/Turkey). The patients were

38–56 years old (mean: 47.73 ± 4.63). The control group individuals were 40–50 years old (mean: 45.65 ± 3.44).

The suitability of the tissues in the patient was determined according to the following inclusion criteria: women in reproductive age, patients in the proliferative period, patients diagnosed with adenomyosis and adenomyosis foci including endometrial gland and stroma (Fig. 1). The suitability of the tissues in the control group was determined according to the individuals without adenomyosis.

After examining the archive samples between 2014 and 2017, 90 samples (patient and control) required for the study were reached. Tissues included in the study were examined histopathologically for compliance with the study criteria, and the diagnosis of these samples was validated. The target tissue foci of the samples that were found to be compatible with the study criteria were marked under the microscope. The labeled regions were then used to remove the correct regions from the sections taken with microtome from paraffin-embedded archival tissue blocks.

This study was approved by the ethics review board of Mersin University.

Reagents

QIAGEN miRNeasy FFPE Kit (Cat. No: 217504) was used for the extraction of total RNA from formalin-fixed paraffin-embedded (FFPE) tissues. Fluidigm D3 Assay Design service was used for the primary design of the real-time assay method. Fluidigm PreAmp Kit (Cat. No: 100-5580), Fluidigm Reverse Transcription Master Mix (Cat. No: 100-6298) and Fluidigm 96.96 Dynamic Array (Cat. No: BMK-M-96.96) were used for gene expression method.
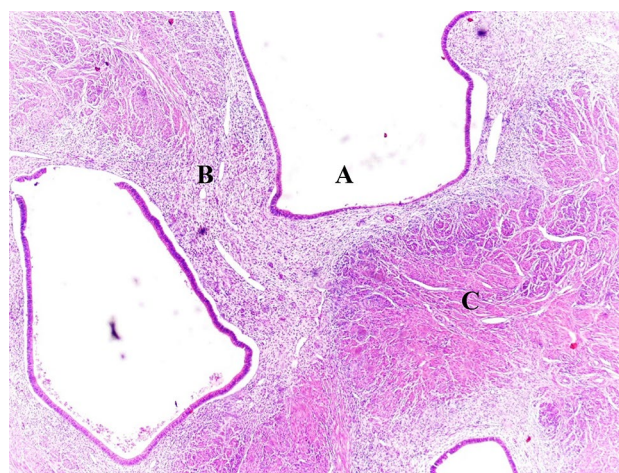

Fig. 1 Adenomyosis focus characterized by cystic enlarged endometrial glands (**a**) and surrounding stroma (**b**) in the myometrium (**c**) (H&E, ×100)

RNA extraction and Rt-qPCR expression array

Initially, xylene was used to remove paraffin from FFPE tissues. After this step, QIAGEN miRNeasy FFPE Kit protocol was applied for total RNA isolation. RNAs isolated from tissue samples were converted to cDNA by the reverse transcription method using Reverse Transcription Master Mix. Reverse transcription reaction program was as follows: 25 °C for 5 min, 42 °C for 30 min and 85 °C for 5 min. According to the Fluidigm PreAmp Kit protocol, preamplification was performed to cDNAs. Preamplification step was as follows: 95 °C for 2 min followed by 14 cycles of 94 °C for 15 s and 60 °C for 4 min. After this stage, the exonuclease protocol was applied by the following reaction conditions: 37 °C for 30 min and 80 °C for 15 min. After the Dynamic Array chip was prepared, samples and primer mix (Forward and Reverse) were loaded into the chip in accordance with the protocol. RT-PCR stage was carried out with BioMark Data Collection software on BioMark HD Real Time Reader. Thermal Cycler Protocol of this step was as follows: 70 °C for 40 min, 60 °C for 30 s, 95 °C for 10 min and 30 cycles of 96 °C for 15 s and 60 °C for 1 min. The study was performed in two repetitions. The GAPDH gene was used as the control gene. Delta Ct (ΔCt) and $2^{-\Delta\Delta Ct}$ values were calculated and used in statistical analysis.

Statistical analysis

Statistical analysis was performed on STATA MP/11 program. Data were expressed as mean and standard deviation. The differences of gene expression levels between the three groups were evaluated by the Kruskal–Wallis test. The Shapiro–Wilk test was used for normal distribution of data. The significance of the differences between the parameters was determined by the post hoc comparison method. $P < 0.05$ was considered to indicate a statistically significant difference. Spearman correlation coefficients were calculated between the parameters of each group in order to see the correlation between the parameters.

Results

Three groups were included in this study: Group I ($n = 35$); ectopic endometrial tissues, Group II ($n = 35$); eutopic endometrial tissues and control group ($n = 20$). The mean age of the Group I and Group II was 47.73 ± 4.63, and the mean age of the control group was 45.65 ± 3.44. The age distribution between the groups was similar ($p = 0.086$), so the results obtained were independent of the age parameter. In the current study, more than 90% of patients were multiparous which is similar to other studies.

Expression of VEGF and HIF-1A

In terms of VEGF (VEGFA) gene expression levels, significant difference was found between Group I–Group II ($p = 0.036$) and Group I–Control Group ($p = 0.001$). No significant difference was found between Group II and Control Group ($p = 0.275$) (Fig. 2). Fold change values were detected as follows; 4.92 between Group I and Group II and 11,983 between Group I and Control Group. This result indicates a significant increase in VEGF expression in the adenomyosis foci compared to the eutopic endometrium and control group.

There was no statistically significant difference in HIF-1A gene expression levels between the groups ($p = 0.535$) (Fig. 3). Although we did not find a significant difference in HIF-1A levels between the study groups, we found a positive correlation between VEGF and HIF-1A in the correlation study of ectopic endometrial tissue samples (Group I) (64%, $p < 0.05$). This shows that the increase in expression of VEGF is due to HIF-1A expression.

Expression of EGF and ICAM-1

There was no significant difference in EGF and ICAM-1 between the groups.

Expression of Bcl-2, Cas3 and Cas9

A significant difference was found between Group I and Group II in terms of Blc-2 gene expression levels
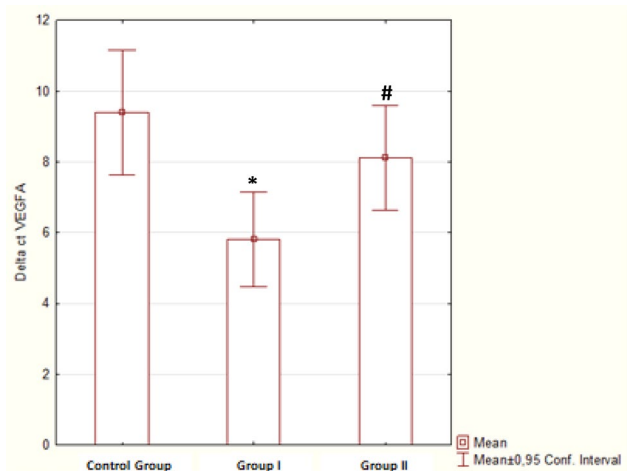

Fig. 2 Comparison of VEGFA ΔCt averages and 95% confidence intervals. *shows differences with Control Group, #shows differences with Group I. $p < 0.05$ statistically significant
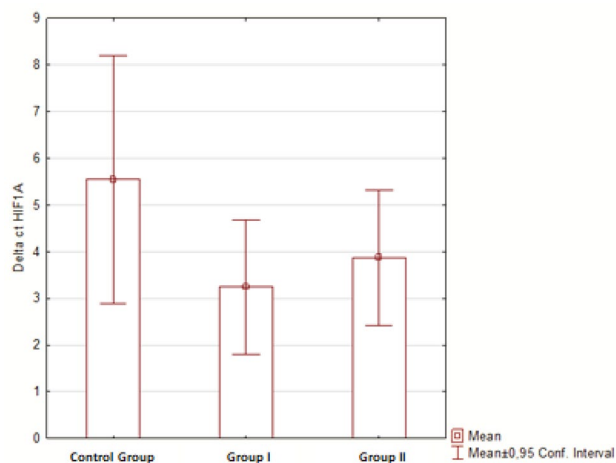
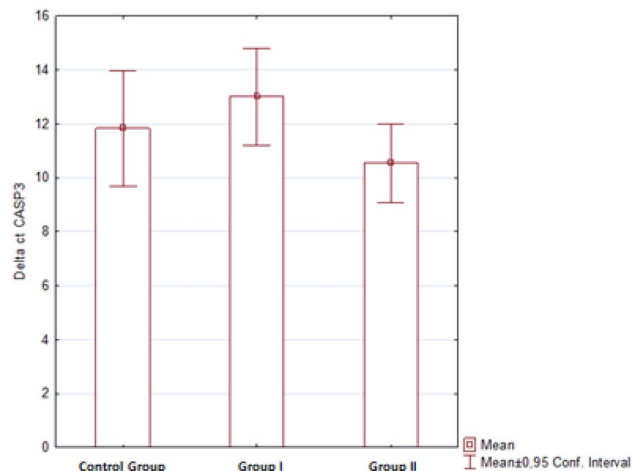**Fig. 3** Comparison of HIF-1A ΔCt averages and 95% confidence intervals

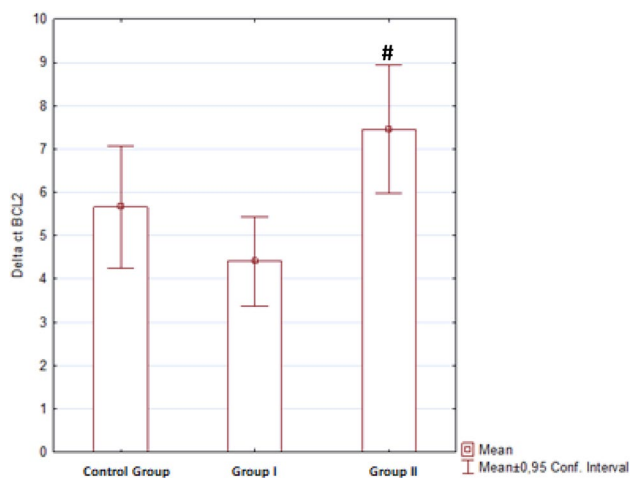**Fig. 5** Comparison of Caspase 3 ΔCt averages and 95% confidence intervals

Fig. 4 Comparison of Bcl-2 ΔCt averages and 95% confidence intervals. #shows differences with Group I. $p < 0.05$ statistically significant

Fig. 6 Comparison of Caspase 9 ΔCt averages and 95% confidence intervals

($p = 0.002$). No significant difference was found between Group I–Control Group ($p = 0.170$) and Group II–Control Group ($p = 0.490$) (Fig. 4). Fold change value was found to be 8.26 between Group I and Group II. This result indicates a significant increase in Bcl-2 expression in ectopic endometrial tissues compared to eutopic endometrial tissues of patients with adenomyosis.

There was no statistically significant difference in Cas 3 and Cas 9 gene expression levels between the groups ($p = 0.099$, $p = 0.893$, respectively) (Figs. 5, 6).

Expression of IDH1

In terms of IDH1 gene expression levels, significant differences were found between Group I–Control Group
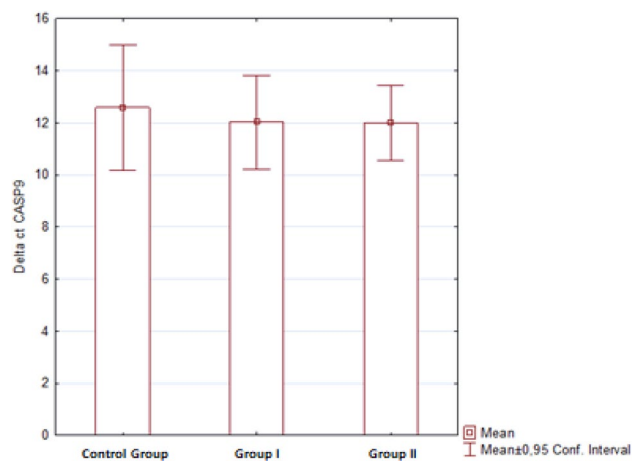
($p = 0.028$) and Group II–Control Group ($p = 0.046$). No significant difference was found between Group I and Group II ($p = 0.337$) (Fig. 7). Fold change values were detected as follows; 5.70 between Group I and Control Group and 3.48 between Group II and Control Group. This result shows a significant decrease in IDH1 expression in the ectopic and eutopic endometrial tissues of adenomyosis patients compared to the control group.

Expression of SDHC and FH

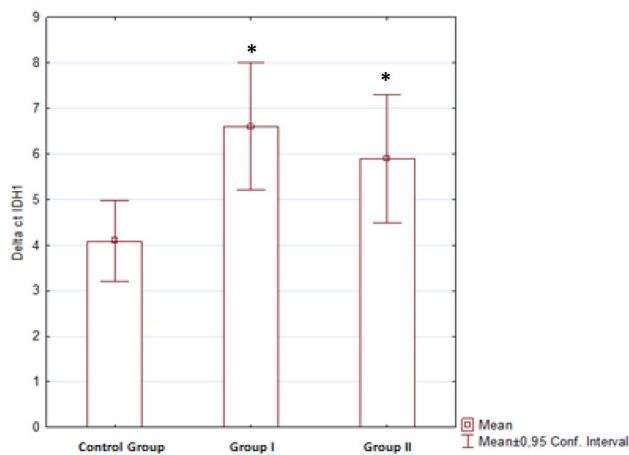There was no significant difference in SDHC and FH between the groups.

Fig. 7 Comparison of IDH1 ΔCt averages and 95% confidence intervals. *shows differences with Control Group. $p < 0.05$ statistically significant

Discussion

Adenomyosis is a myometrial lesion, which is characterized by the presence of ectopic endometrial glands and stroma in myometrium, affecting the reproductive period of life in women [3, 4, 19]. It is usually diagnosed in multiparous patients over 30 years of age [2]. Although it is a common disease, the etiology and pathogenesis of adenomyosis remain unclear [3, 4].

Previous studies have shown that some genes are expressed differently in adenomyosis [20]. The examinations with microarray and immunohistochemical staining methods have been shown the genes associated with apoptosis and angiogenesis such as Bcl-2 and VEGF expressed differently in adenomyosis.[21, 22]. These molecular differences suggest that angiogenesis and apoptosis are involved in the myometrial invasion of the eutopic endometrium and pathogenesis of adenomyosis [16, 21, 22].

Abnormal vascularization was detected in adenomyosis patients compared to healthy women [2, 14]. VEGF, one of the major mediators of angiogenesis, is thought to be a primary factor in this process. VEGF expression levels have been shown to change in adenomyosis [15, 23]. VEGF increases vascular permeability and is therefore thought to play an important role in the development of adenomyosis [15, 16].

Eutopic and ectopic endometrial tissues of patients with adenomyosis were shown to have higher VEGF expression compared to the endometrial tissues of the healthy control group [15, 22]. In the current study, a significant VEGF expression increase was found in ectopic endometrial tissues compared to both the eutopic endometrial tissues and the control group. This result is similar to previous studies and suggests that VEGF may play a critical role in the development of adenomyosis. This increase in VEGF supports the idea that angiogenic processes can be activated via VEGF in order to increase vascularization of the myometrium in patients with adenomyosis.

One of the most important transcription factors of VEGF in angiogenesis is HIF-1A. Although we did not find any significant HIF-1A expression difference between the groups, we found a positive correlation between VEGF and HIF-1A as a result of the correlation study in ectopic endometrial tissue samples. This result suggests that the increase in the expression of VEGF in adenomyosis tissue may have been via HIF-1A. Goteri et al. did not find a significant HIF-1A expression increase in the eutopic endometrium of patients with adenomyosis compared to the control group. However, a significant increase in VEGF expression was found in the same group [15].

Continuous expression of Bcl-2, an anti-apoptotic molecule in adenomyosis, has been suggested to promote endometrial invagination to myometrium [11]. We found a significant increase in Bcl-2 expression in the ectopic endometrial tissues of the adenomyosis patients compared to the eutopic endometrial tissues of the same patients. This result suggests that Bcl-2 may cause the formation of adenomyotic foci of the endometrial cells in the myometrium by supporting escape from apoptosis. There was no significant difference and increase in expression levels of caspase-3 and caspase-9 between the groups. Increased expression of Bcl-2 and no significant change in caspase 3 and caspase 9 levels between the groups indicate that apoptotic processes were suppressed in adenomyosis. Despite these results, the arguments regarding the role of apoptosis in adenomyosis are insufficient and more studies are needed.

Under physiological conditions, cells benefit from the oxidative phosphorylation in mitochondria to produce the energy they need. During the uncontrolled cell proliferation and tumoral development process, changes occur in energy metabolism [24]. Some of these are found in mitochondria where a significant portion of the energy production takes place. The enzymes of the krebs cycle play an important role in the changes in energy metabolism [25]. Endometriosis is a benign gynecologic disease characterized by the presence of the growth of uterine endometrium outside of the uterus. A study in nonhuman primates about endometriosis has shown that mitochondrial energy metabolism is reduced in endometrium and endometriosis tissue. However, the exact cause of this shift of mitochondrial metabolism remains unclear [26]. The current study is the first study to investigate the IDH1 enzyme level in adenomyosis. At the end of the experimental process, a significant difference was observed in IDH1 between the study groups. We observed that endometrial cells (both ectopic and eutopic) of adenomyosis patients expressed lower levels of IDH1 than endometrial cells of the control group. In adenomyosis patients, IDH1 levels

were found to be significantly lower in ectopic and eutopic endometrial tissues compared to the control group. These results show that IDH1 may be an effective factor in the etiology of adenomyosis. The current study is the first IDH1 report in the literature about adenomyosis. We believe that this report related to IDH1 will lead to future studies about energy metabolism in adenomyosis. In addition, molecular processes investigated in the current study could be a potential therapeutic and diagnostic goal for adenomyosis patients and the results could be a reference for further studies about the molecular mechanism of adenomyosis. Studies on the molecular mechanism of adenomyosis will lead to the discovery of more effective treatment and diagnostic options.

Conclusion

In conclusion, the current study shows that angiogenic processes are activated and apoptotic processes are suppressed in adenomyosis and also the importance of IDH1 a member of the Krebs cycle in energy metabolism in adenomyosis. These results suggest that adenomyosis mimics the malignant process in terms of angiogenesis, apoptosis, hypoxia and energy metabolism, although it does not have a malignant potential.

Compliance with ethical standards

Conflict of interest No authors have any financial/conflicting interests to disclose.

Ethical approval This study, which is the part of ''Investigation of Angiogenesis, Apoptosis and Energy Metabolism in Adenomyosis'' project, was approved by the Ethics Committee of Mersin University (Mersin, Turkey) (13/04/2017, 93).

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