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Relevance of lipogenesis and AMPK/Akt/mTOR signaling pathway in endometrial cancer

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Abstract. – OBJECTIVE: New evidence is presented for differences in lipid metabolism between healthy and cancer cells, which has increased considerably in recent years. Alterations in lipid metabolism affect important processes such as cell growth, proliferation, and differentiation. Therefore, the purpose of this study is to determine the gene expression levels of the enzymes which have a function in lipid metabolisms such as fatty acid synthase (FASN), sterol regulatory element-binding protein (SREBP), phosphatidylinositol 3-kinase (PI3K), mammalian target of rapamycin (mTOR), protein kinase B (Akt), and AMP-activating protein kinase (AMPK) in patients with endometrial cancer.

PATIENTS AND METHODS: This study included 60 patients diagnosed with endometrial adenocarcinoma which subgrouped as Grade 1 (n = 20), Grade 2 (n = 20), Grade 3 (n = 20). For control group endometrial tissues from 30 individuals subgrouped as control 1 (n = 15) secretory phase and control 2 (n = 15) proliferative phase healthy endometrial tissues. Gene expression analysis was performed using Real-time polymerase chain reaction (RT-PCR).

RESULTS: PI3K gene expression levels were decreased in patients with Grade 3 endometrial cancer compared to Grade 1 and 2 ($p < 0.05$). The gene expression levels of Akt and mTOR were decreased in the Grade 3 patients compared to control groups. The gene expression levels of SREBP, FASN, and AMPK were decreased in all patients' groups compared to control groups ($p < 0.05$).

CONCLUSIONS: The results suggest that while lipogenesis may show different tissue-specific behaviors related to some pathways, it may have a direct relationship with endometrial cancer.

Key Words:
Endometrial cancer, Lipogenesis, SREBP, PI3K/Akt/mTOR, FASN, AMPK.

Introduction

Endometrial cancer is the most common malignancy of the female reproductive system. Every year, 320,000 new cases are diagnosed globally, and 76,000 patients die worldwide due to endometrial cancer[1,2]. Traditionally, endometrial cancer has different clinical, pathological, histological, and molecular behavior and is divided into two subgroups, Type 1 and Type 2[3,4].

Type 1 endometrial cancers are low grade, estrogen-dependent, hormone receptor-positive adenocarcinomas with endometrioid morphology and are often referred to as endometrioid endometrial cancers. Type 1 endometrioid cancers account for 85% of all endometrial cancers. They are often diagnosed at an early stage and are usually characterized by a good prognosis[1,5]. Type 2 endometrial cancers are serous and clear cell carcinomas that are aggressive types, affecting about 10-15% of diagnosed patients[6]. The most important risk factors of endometrial cancer are increased estrogen, use of tamoxifen, obesity, age, early menstruation, and late menopause[7].

Changes in lipid metabolism, known as an energy-providing molecule, have great importance in cancer development. The increase of fat mobilization in cancer cells causes hyperactivity in fatty acid metabolism, which in turn increases plasma-free fatty acid concentration due to fasting, insufficient glucose use, prolonged malnutrition, secretion of adrenaline, and growth hormones[8]. The increased proliferation of cancer cells requires acceleration of lipid synthesis for the construction of biological membranes as these energy-rich lipids provide energy to cancer cells in nutrient deficiency[9].

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One of the most studied lipid metabolism enzymes against cancer is FASN[10]. The SREBP molecule is another important transcription regulatory factor in pathway[11]. In the control of cellular metabolism, the PI3K pathway functions particularly in glucose transport and utilization, regulation of cell growth, protein biosynthesis, and prevention of apoptosis. Akt regulates cellular metabolism through various sub-targets[12]. mTOR plays an important role in cell growth and metabolism[13]. The irregularity of the PI3K/AKT/mTOR signal pathway plays a role in the pathogenesis of many types of cancer. AMPK, originally known as serine/threonine kinase, regulates the negative regulation of many key enzymes of lipid anabolism. Over time, AMPK has been simultaneously inhibited by many anabolic pathways such as lipids, carbohydrates, protein biosynthesis, while being considered an energy sensitive kinase that activates catabolic pathways in various multicellular organisms such as glucose uptake and metabolism[14].

Obesity, one of the main risk factors of endometrial cancer, can be examined to emphasize the importance of its related molecules and pathways in endometrial cancer. In obesity, cancer cells are constantly stimulated by proinflammatory cytokines and adipokines, among which leptin is dominant. Studies indicate that leptin can affect cancer cells through some phenomena, including inflammation, cell proliferation, suppression of apoptosis, and angiogenesis[15]. Increased leptin in obesity is an adipokine that contributes to hyperestrogenemia and the growth of estrogen-dependent tumor cells[16]. Many studies have associated leptin with activation of the mTOR pathway. Metformin, which is effective in the treatment of endometrial cancer, has been evaluated as a therapeutic agent due to the inhibition of the P13K/Akt/mTOR pathway and thus indirect inhibition of downstream leptin signaling. Metformin directly activates AMPK, which then phosphorylates the tuberous sclerosis 2 protein and subsequently inhibits the mTOR signal, leading to a decrease in cell proliferation. Metformin also indirectly affects cell growth by increasing insulin sensitivity, resulting in increased intracellular glucose uptake and decreased peripheral insulin levels[17].

Early diagnosis of endometrial cancer is highly possible, and treatment offers positive results. The ability to treat cancer without reducing the quality of life by eliminating or controlling the causes of cancer is the target of cancer pathway studies[18].

In this study, it was aimed to determine the levels of gene expressions of FASN, SREBP, PI3K, Akt, mTOR, and AMPK in patients with endometrial cancer. According to our results, it was reported that lipogenesis and PI3K/Akt/mTOR signaling pathway mediators may play a role in the diagnosis, treatment, and prognosis of endometrial cancer.

Patients and Methods

Patients and Tumor Samples

Prospectively collected (2012-2018) formalin-fixed paraffin-embedded (FFPE) tissue specimens' series were retrieved from Mersin University Faculty of Medicine, Department of Obstetrics and Gynecology. All of these samples were linked to comprehensive clinical, histologic, and molecular data.

A total of 60 patients diagnosed with endometrioid adenocarcinoma were included in this study. All patients were divided into 3 groups, which included 20 patients per group, according to the tumor grades. Besides, two control groups were also included: Control 1 with 15 proliferative samples, and Control 2 with 15 secretory phase healthy samples.

Collecting Samples from Paraffin Archive Tissues

FFPE tissues were prepared using the modified tissue microarray method for analysis. With the modified tissue microarray method, samples from different areas of the same tumor can be taken and evaluated, and tissue samples of different cases can be examined together in the same paraffin block. For this purpose, in the present study, a punch biopsy device designed for the removal of skin biopsies was used in the preparation of the blocks.

Appropriate areas on preparations without detection-tracking artifacts and with little or no necrosis were marked using a light microscope. These areas were selected from 4 different blocks. A tissue sample of 4 mm in diameter was removed from the paraffin block from areas corresponding to those marked on the preparations. New paraffin blocks were obtained by mapping 5 different cases on one block, with 4 tissues extracted from different blocks of the same case on the same line. Each block was created from 20 textures containing 5 rows and 4 columns. From these newly prepared blocks and control blocks, microtome sections with a thickness of 15 μm were taken,

and the sections were transferred onto clean slides (Figure 1).

Primer Design

National Center for Biotechnology Information database was used to design the primers for the genes which were performed in this study. The designability report of the determined primers was received via the "FLUIDIGM D3 Assay Design" service.

Deparaffinization and RNA Isolation

Sample separation from the paraffin block was performed according to the QIAGEN miRNeasy FFPE Kit (Cat No: 217504, Hilden, Germany) protocol. After the necessary procedures were carried out, the total RNA for cDNA extraction was filtered into the tube.

cDNA Synthesis, Preamplification, and RT-PCR Application

Reverse Transcription Master Mix (P/N: 100-6298, South San Francisco, CA, USA) was used to convert RNAs isolated from tissue samples into cDNA using the Reverse Transcription method according to the "Fluidigm cDNA production" protocol. After the cDNA was synthesized, the preamplification phase was initiated. The Fluidigm PreAmp Kit (Fluidigm, 100-5580, South San Francisco, CA, USA) protocol was thus applied. The Dynamic Array chip was placed on the BioMark HD Real-Time Reader. RT-PCR was carried out using BioMark Data Collection software.

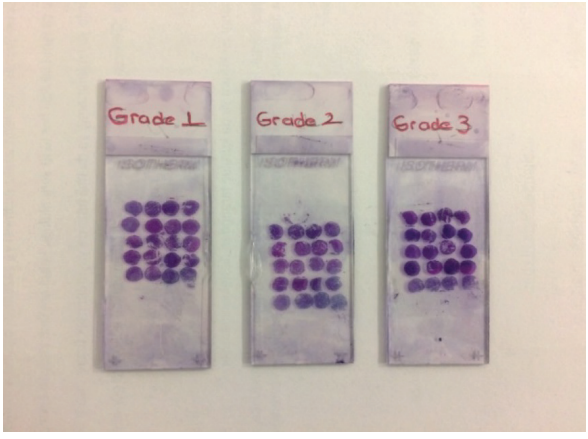

Figure 1. Determination of target tissues by marking in archive tissue preparations.

Statistical Analysis

Statistical analysis was performed with Free Trial IBM SPSS statistical software (SPSS Inc., Chicago, IL, USA). In the analysis of the study results, the delta ct values were calculated based on the difference between the ct values of the target and reference genes. (Δct). Δct values were calculated using the Pfaffl method formula[19].

Data were controlled for normal distribution using the Shapiro-Wilk test. According to the test results, while Control 1 and Control 2 groups showed a normal distribution ($p > 0.05$), the Grade 1, Grade 2, and Grade 3 groups did not ($p < 0.05$). The Kruskal Wallis multiple comparisons and Kruskal Wallis Dunn's post hoc tests were used to detect differences between groups in terms of gene expressions. For all analyses, a p-value <0.05 was regarded as statistically significant.

Ethics Committee Approval

This study, as part of the "Relation between lipogenesis and AMPK/Akt/mTOR in Endometrial Carcinoma" project, was approved by the Ethics Committee of Mersin University (Mersin, Turkey) (21/09/2017, 16/274).

Results

This study included 60 patients with endometrioid adenocarcinoma as well as 30 healthy subjects. The FASN, SREBF1, SREBF2, PIK3CA, AKT1, MTORC1, PRKAA1, PRKAA2, and PRKAB1 expression profiles were determined. GAPDH is one of the most common housekeeping genes and was used as an endogenous control in the quantitative analysis of RT-PCR to normalize the distinguishing expression of tissues.

Participants who have similar ages were included in this study. The mean age of the patient group was 62.67 ± 11.082, while the mean age of the control group was 42.90 ± 4.163. Prognostic factors such as myometrial invasion, distance to serosa, age, and lymph node involvement were compared between the Grade 1, Grade 2, and Grade 3 groups (Table I).

The Grade 1, Grade 2, and Grade 3 groups were compared in terms of myometrial invasion, distance to serosa, age, and lymph node involvement. Results showed that there was a significant difference in myometrial invasion between the groups ($p = 0.004$). Myometrial invasion was found to differ significantly between Grade 1 and Grade 2 ($p = 0.005$), and Grade 1 and Grade 3

Table I. Grade 1, Grade 2, Grade 3 prognostic factors.

	Grade 1 (mean ± SEM) (n: 20)		Grade 2 (mean ± SEM) (n: 20)		Grade 3 (mean ±SEM) (n: 20)		*P*
Myometrial invasion	0.435±0.114		1.065±0.149 [a]		1.01±0.168 [a]		0,004
Distance to serosa	0.620±0.125		0.797±0.207		0.565±0.131		0.814
Lymph node involvement	**Positive (n, %)**	**Negative (n, %)**	**Positive (n, %)**	**Negative (n, %)**	**Positive (n, %)**	**Negative (n, %)**	*p*
	4, 20%	16, 80%	1, 5%	19, 95%	2, 10%	18, 90%	0.322

Results are expressed as mean ± SEM. SEM; standard error rate. aShows statistically significant difference (p <0.05) with Grade 1 group.

(p = 0.028). There was no significant difference between the groups in terms of lymph node involvement and distance to serosa.

Results of Lipogenesis Markers

FASN expression levels were found significantly different between patient and control groups (p = 0.034). It was determined that FASN expression decreased in patient groups when compared to the control groups. A significant difference was determined in the Grade 2 group compared to the Control 2 group (p = 0.028) (Figure 2 A).

Furthermore, the expression levels of SREBF1 gene, which stimulate fatty acid synthesis, were found significantly different compared to other groups (p = 0.0001). There was a significant difference between Grade 2 and Control 1 (p = 0.012) and between Grade 3 and control 1 (p = 0.0001) (Figure 2 B).

Besides, SREBF2, one of the cholesterol metabolism stimuli gene expression, showed a significant difference between the groups (p = 0.0001). SREBF2 expression levels were significantly decreased in the patient group when compared

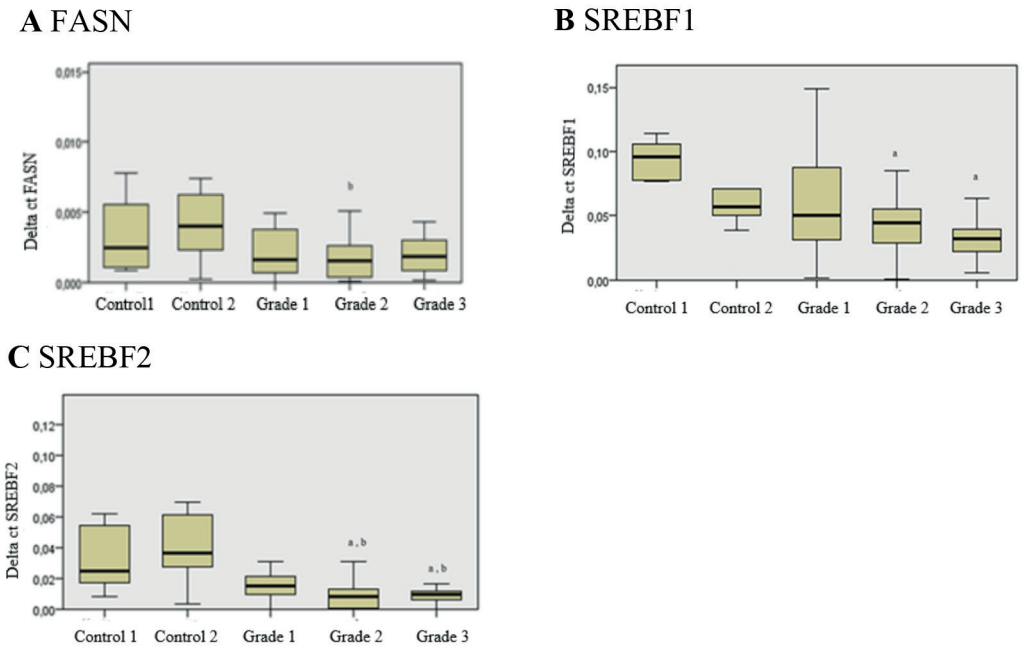

Figure 2. Results of Lipogenesis Markers **A,** Gene expression of FASN, **B,** Comparison of SREBF1 delta ct values. **C,** Comparison of SREBF2 delta ct values. [a]shows differences between control 1, [b]shows differences between control 2. p <0.05 statistically significant.

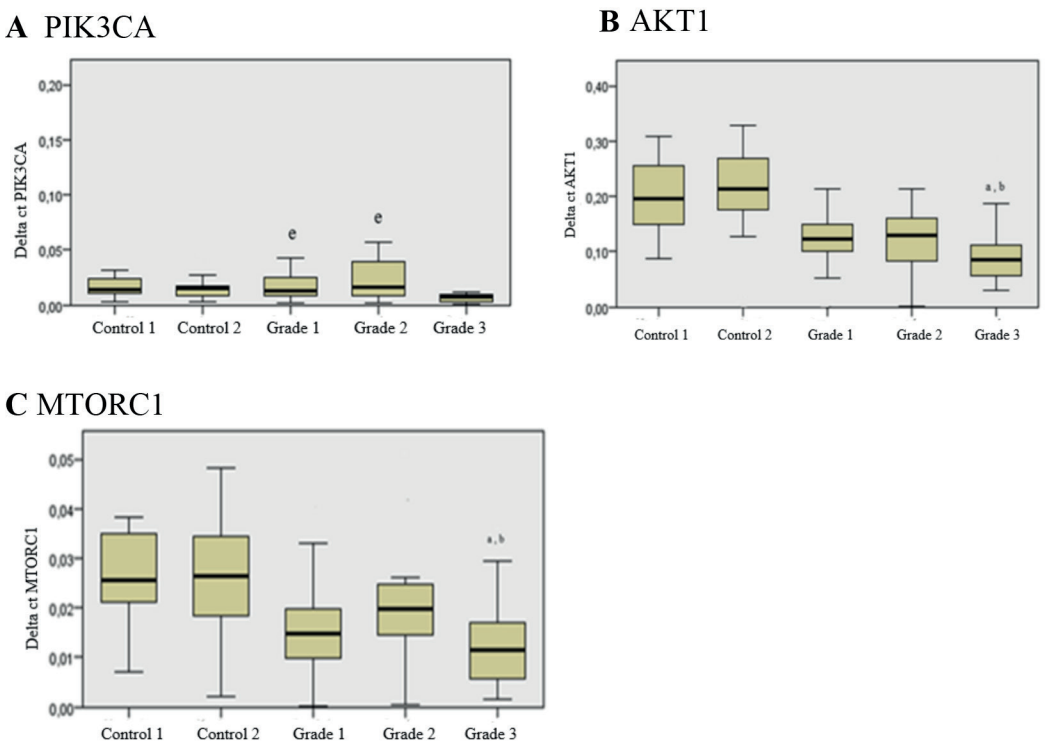

Figure 3. Results of PI3K/Akt/mTOR signal transmission pathway. **A,** Comparison of PIK3CAdelta ct values. **B,** Comparison of AKT1 delta ct values. **C,** Comparison of MTORC1 delta ct values**.** [a] shows differences between control 1, [b] shows differences between control 2, [e] shows differences between grade 3. $p<0.05$ statistically significant.

to the control group Grade 2 and Control 1 (p = 0.002) and Grade 2 and Control 2 (p = 0.0001), respectively. Similar significant differences were determined between Grade 3 and Control 1 (p = 0.003) as well as Grade 3 and Control 2 (p = 0.0001) (Figure 2 C).

Results of PI3K/Akt/mTOR Signal Transmission Pathway

When PIK3CA gene expression levels were compared, a significant difference was found between the groups (p = 0.002). Although it was not statistically significant, the expression levels of PIK3CA gene in the Grade 1 and Grade 2 groups were higher than the control groups. On the other hand, the expression levels of PIK3CA gene in Grade 3 group showed a significant decrease compared to the Grade 1 (p = 0.014) and Grade 2 (p = 0.003) groups. This result can be explained by the fact that PI3K increases as expected in the early stages of cancer and decreases as the tumor progresses (Figure 3 A).

It was shown that the Akt1 expression level was significantly different between the groups (p = 0.0001). There was a significant difference between Grade 3 and Control 1 (p = 0.002) as well as Grade 3 and Control 2 (p = 0.001) (Figure 3 B). AKT1 expression levels of patient groups decreased compared to control groups but this reduction was not statistically significant.

When we compare the gene expression levels of MTORC1 complex as a continuation of the signaling pathway a significant difference was found between groups (p = 0.001). Grade 3 compared to the Control 1 (p = 0.005) and Control 2 (p = 0.015) groups, respectively (Figure 3 C). There was a decrease in MTORC1 expression levels in patient groups when compared to control groups, but this decrease was not statistically significant.

Results of AMPK Subunits

The alpha and beta subunits of AMPK, known as energy metabolism sensors and regulators, provide AMPK activation. In this study, the expression levels of AMPK alpha 1 (PRKAA1), alpha 2 (PRKAA2), and beta (PRKAB1) subunits in both the patient and control groups were analyzed. Significant decrease in PRKAA1 expression levels

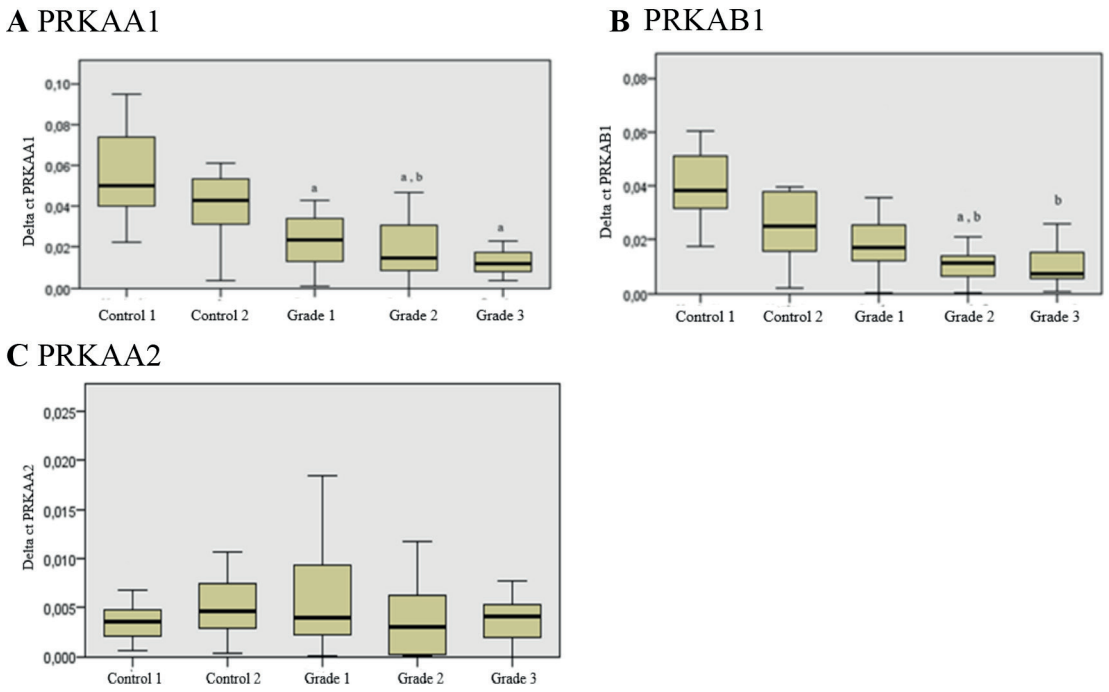

Figure 4. Results of AMPK subunits. **A,** Comparison of PRKAA1 **d**elta ct values. **B,** Comparison of PRKAB1 delta ct values. **C,** Comparison of PRKAA2 delta ct values. [a] shows differences between control 1, [b] shows differences between control 2. $p<0.05$ statistically significant.

was observed in Grade 1 compared to Control 1 ($p = 0.022$), Grade 2 compared to Control 1 and Control 2 ($p = 0.003$), and Grade 3 compared to Control 1 ($p = 0.0001$) (Figure 4 A).

PRKAB1 acts as a bridge between AMPK subunits. It was shown that a significant decrease was observed in expression levels in Grade 2 ($p = 0.001$) compared to Control 1 and Control 2, and in Grade 3 compared to Control 2 ($p = 0.034$) (Figure 4 B).

There was no significant difference between the groups in terms of PRKAA2 expression results. ($p = 0.603$) (Figure 4 C).

Discussion

Endometrial cancer is a gynecological cancer that ranks fourth in developed countries, and its prevalence keep increasing annually[1]. Lipid metabolism, which provides extra energy sources for metastasis and proliferation and can act as a secondary messenger in various signaling pathways, is now recognized as an important metabolic pathway in cancer. FASN is an important molecule in the lipid metabolic pathway and has the ability to rewire tumor cells to provide greater energy flexibility to achieve high energy requirements[20].

FASN catalyzes the condensation of acetyl-CoA and malonyl-CoA to produce long-chain fatty acids[12]. In this study, it was observed that FASN expression decreased in the patient group compared to the control group. Although FASN expression increases in endometrial glands and stromal cells from the proliferative period to the early secretory phase, it decreases after the cessation of cell proliferation in the late secretory phase[21,22].

SREBF1 gene expression, which stimulates fatty acid synthesis, was found to be downregulated in the patient when the patient and control group results were compared. Studies have shown that downregulation of SREBF1 expression inhibits cell growth, migration, and invasion and induces apoptosis in ovarian cancer cells[23]. Similarly, evaluation of SREBF2, one of the stimuli of cholesterol metabolism, indicated a decrease in the patient group compared to the control group.

The observation of increased SREBF1 expression and activation in the proliferative phase is consistent with the idea that proliferative cells in

the endometrium require de novo lipogenesis. In addition, the SREBF1 protein was not detected in most of the postmenopausal endometrium and stromal cells independent of endometrial phases[24].

The PIK3CA gene encodes the catalytic subunit p110 alpha of the PI3K enzyme and activating mutations of PIK3CA have been frequently identified in many cancers. These mutations were found to produce the active PI3K form resulting in high protein activities. Activating mutations of PIK3CA have been associated with the growth of epithelial cells and cancer[25].

As a result of this study, a significant difference was found between the groups in terms of PIK3CA gene expression levels. Although it was not statistically significant, it was determined that the expression levels of Grade 1 and Grade 2 of the patient groups were higher than the control groups, and the Grade 3 group showed a significant decrease compared to the Grade 1 and Grade 2 groups. These results suggested that PIK3CA is active in the initial stages of the tumor with the effect of different factors but does not show the same activation in the later stages of the tumor.

It was reported that in a previous research PIK-3CA activates mutations in endometrial cancer. Velosco et al[26] detected PIK3CA mutations in 24% of their endometrial cancer samples. Konopka et al[27] detected 20% mutation and 12.2% amplification in 196 endometrial carcinoma samples. Oda et al[28] investigated PTEN and PIK3CA gene mutations and detected PIK3CA mutation in 36% and both PTEN and PIK3CA mutations in 26% of 66 endometrial cancer patients.

The PI3K-Akt-mTOR pathway stimulates the proliferation, growth, and survival of cancer cells. It also controls anabolic and catabolic processes and supports aerobic glycolysis (the Warburg effect) and lipid biosynthesis, which are characteristic of neoplastic cells[29].

PKB, also known as evolutionarily conserved serine/threonine kinase Akt, is one of the most commonly activated protein kinases in cancer. Hyperactivation of Akt is associated with apoptosis resistance, increased cell growth, cell proliferation, and cellular energy metabolism[30]. The connection of the PI3K-Akt pathway with lipogenesis was supported by *in vitro* studies[31].

AKT gene expression levels were found significantly different between the groups. In particular, the decrease determined in the Grade 3 group compared to the control groups suggests that the progression of the tumor is inversely proportional to the AKT expression level.

A significant decrease was found between groups in gene expression of MTORC1 complex. Although an increase is expected in PI3K and its downstream targets, Akt and mTOR molecules, which are activated in cancer, in parallel with the process, different results have been reported from recent studies. In the endometrial cancer research conducted by Holst et al[32], the relationship between PIK3CA mutations (exons 9 and 20), gene expression, PIK3CA amplification from the same patients, and clinical, histological, and molecular data were analyzed. PIK3CA amplification was associated with some features of PI3K pathway activation, but it was not associated with determinants of Akt and mTOR activity.

The decrease of AKT1 and MTORC1 markers in Grade 3 compared to the control groups can be interpreted as suppression of the Akt/mTOR pathway in the advanced stages of the disease. This decrease in SREBF1 and SREBF2 levels in the patient groups may be due to the low mTOR and Akt levels in the patient groups.

Two important tumor suppressor factors have been reported in the PI3K/Akt/mTOR pathway. These are PTEN, which acts as a brake upstream of Akt, and a TSC1/TSC2 heterodimer that acts as a brake downstream of Akt and upstream of mTOR. MTOR, which is activated in the absence of the TSC1/TSC2 brake, operates as a feedback mechanism inhibiting Akt30.

Evaluation of previous research indicates that the PI3K/Akt/mTOR pathway is important in cancer development; however, it does not show the same activation in every tissue type or every cancer process. Due to the fact that pathway members are activated or inactivated independently each other leads to the need for different studies to evaluate these members as cancer markers.

AMPK is a serine/threonine protein kinase and is known as the "intracellular energy sensor and regulator". Downregulation of AMPK activity is associated with tumorigenesis. Several mechanisms contribute to decreased AMPK activity in tumor formation, including self-inhibition, post-transcriptional, and post-translational modification[33]. AMPK activity is lower in various tumor tissues than neighboring para tumor or normal tissues and further decreases with progressive tumor stage[34]. In ovarian cancer, AMPK β1 mRNA and protein expression, as well as AMPK activity, have been found to gradually decrease with malignant progression of the cancer[35].

According to the results obtained, the AMPK expression level was found to be lower in the pa-

tient groups compared to the control groups. This decrease in the patient groups continued with the grade of the tumor. It has been observed that the expression level of AMPK is low in endometrial cancer tissues, like the studies performed in other tumor tissues. On the other hand, the decrease observed in the expression levels of SREBP, FASN, mTOR, which are among the target genes that AMPK inhibits, suggests that AMPK does not show the same effect on endometrial tissue as on other tissues.

Conclusions

In line with these results as well as previous research, it is suggested that lipogenesis has a direct relationship with endometrial cancer, but it may exhibit different tissue-specific behavior with some pathways.

Therefore, the efficient PI3K/Akt/mTOR signaling pathway in lipogenesis and SREBP1-2, FASN, AMPK genes affected by this pathway would benefit from further detailed studies. Investigation of the metabolism and signal transduction pathways may help in the development of therapeutic strategies for endometrial cancer treatment.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgments

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