Research Article / Araştırma Makalesi

Immunohistochemical Investigation of P16 Expression in Curettage Biopsies

Endometrial Küretaj Olgularında p16 Ekspresyonunun İmmünohistokimyasal Olarak İncelenmesi

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Abstract: Our study aims to investigate the immunohistochemical expression of the P16 molecule, which is involved in the cell cycle and plays a role in developing endometrial cancer in normal epithelium, endometrial polyp, and precursor lesions. A total of 68 patients underwent endometrial sampling for various reasons at the Department of Obstetrics and Gynecology, Faculty of Medicine, Kafkas University, between 2020 and 2021 were included in the study. The selected cases were categorized into four groups: proliferative endometrium, endometrial hyperplasia without atypia, atypical hyperplasia / endometrioid intraepithelial neoplasia (AH / EIN) and endometrial polyp. There were no cases with a diagnosis of endometrial tumors in our study. All patients' pathology samples were re-evaluated, and P16 immunohistochemistry was applied to tissue samples. Among patients diagnosed with atypical endometrial hyperplasia, 72.7% exhibited moderate P16 protein expression, 18.2% had low expression, and 9.1% had high protein expression. The number of patients diagnosed with AH / EIN had a very low frequency in the study population. Among patients diagnosed with endometrial polyps, 50.0% showed moderate P16 protein expression, 20.0% exhibited low protein expression, and 30.0% had high protein expression. High P16 expression has been reported to be significantly associated with endometrial cancer in the literature. P16 expression is significant in precancerous lesions and stages of cancer development. Larger-scale studies with more cases are needed in this regard.
Keywords: Expression, Endometrial biopsies, Endometrial cancer, Endometrial hyperplasia, Endometrial polyp, P16

Özet: Çalışmamız, hücre döngüsünde yer alan ve endometrium kanseri gelişiminde rol oynayan P16 molekülünün normal epitel, endometrial polip ve prekürsör lezyonlardaki immünohistokimyasal ekspresyonunu araştırmayı amaçlamaktadır. Çalışmaya 2020-2021 yılları arasında Kafkas Üniversitesi Tıp Fakültesi Kadın Hastalıkları ve Doğum Anabilim Dalı'nda çeşitli nedenlerle endometriyal örnekleme yapılan 68 hasta dahil edildi. Seçilen vakalar dört gruba ayrıldı: proliferatif endometriyum, atipisiz endometrial hiperplazi, atipik endometrial hiperplazi/endometrioid intraepitelyal neoplazi ve endometrial polip. Çalışmamızda endometrial tümör tanısı alan olguya rastlanmadı. Tüm hastaların patoloji örnekleri yeniden değerlendirildi ve doku örneklerine P16 immünohistokimyası uygulandı. Atipik endometriyal hiperplazi tanısı alan hastaların %72,7'sinde orta derecede P16 protein ekspresyonu, %18,2'sinde düşük ekspresyon ve %9,1'inde yüksek protein ekspresyonu gösterdi. Atipik endometrial hiperplazi tanısı alan hasta sayısı çalışma popülasyonunda çok düşük bir sıklığa sahipti. Endometriyal polip tanısı alan hastaların %50,0'ı orta derecede P16 protein ekspresyonu gösterdi, %20,0'ı düşük protein ekspresyonu gösterdi ve %30,0'ı yüksek protein ekspresyonu gösterdi. Literatürde yüksek P16 ekspresyonunun endometrium kanseri ile anlamlı derecede ilişkili olduğu bildirilmektedir. P16 ekspresyonu kanser öncesi lezyonlarda ve kanser gelişiminin aşamalarında önemlidir. Bu konuda daha büyük ölçekli, daha fazla olgu içeren çalışmalara ihtiyaç vardır.
Anahtar Kelimeler: Endometriyum kanseri, Ekspresyon, Endometriyal örnekleme, Endometriyal hiperplazi, Endometriyal polip, P16

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1. Introduction

P16 is the most commonly used immunohistochemical marker in gynecopathology cases (1). HPV-related, high-risk precancerous lesions show strong immunoreactivity. This immunoreactivity can routinely distinguish benign and malignant lesions (2). P16 is now routinely used as a differential marker in sensitive cases such as endometrial intraepithelial carcinoma and serous adenocarcinoma (3). There are very few studies on the immunoreactivity of p16 in epithelial cells (4, 5). P16 is encoded by the INK4a/ARF locus, a gene located on chromosome 9p21(6). P16 is an important marker due to its high expression in pre-tumor lesions (7). P16 expression in tumor cells occurs by two different mechanisms (8). The first of these begins with an abnormality in the Rb pathway. According to the first mechanism, P16 blocks Rb phosphorylation by inhibiting CDK4/6 (9). In this case, P16 loses its function and allows it to proliferate uncontrollably (10). Another situation, according to the first mechanism, is this: P16 is sometimes expressed at high levels in some malignant tumors unrelated to HPV (11). In this second mechanism, HPV oncogene E7 inactivates Rb, causing uncontrolled release and increased expression of P16 (12). The second mechanism occurs through oncogene-induced aging (13). P16 initiates cellular senescence by arresting the cell cycle to respond to oncogenes (14). This mechanism is also observed in certain benign tumors, such as neurofibromas and schwannomas. Tumors with this mechanism overexpress P16, inhibiting acidic β-galactosidase activity, cell cycle, and BRAF mutation (15). Immunohistochemical analysis reveals intense P16 expression in benign tumors, contrasting with its negative expression in malignant ones. This situation indicates P16's role in safeguarding tumor cells against malignant transformation through proliferation control. Our study aims to investigate the immunohistochemical expression of the P16 molecule, which is involved in the cell cycle and plays a role in developing endometrial cancer.

2. Materials and Method

Our study received ethical approval from the Faculty of Medicine Ethics Committee, Kafkas University, on November 23, 2021, with reference number 80576354-050-99/231.

Study Design and Inclusion Criteria: The study included 68 patients who underwent endometrial sampling for various reasons at the Department of Obstetrics and Gynecology, Faculty of Medicine, Kafkas University, between 2020 and 2021. Due to its retrospective nature, patients with incomplete records and problematic preparations were excluded from the study.

2.1. Tissue specimens

The patients were divided into four groups based on the pathology diagnosis: proliferative endometrium, endometrial hyperplasia without atypia, atypical endometrial hyperplasia, and endometrial polyp. Of these, 20 were diagnosed with proliferative endometrium, 33 with hyperplasia without atypia, 2 with atypical endometrial hyperplasia, and 13 with endometrial polyps. There were no cases diagnosed with endometrial tumors.

2.2. Histopathological examination

The curetted or resected specimens were fixed in 10% neutral-buffered formalin and embedded in paraffin blocks. From each formalin-fixed, paraffin-embedded block, 4-µm sections were cut and stained with hematoxylin and eosin.

2.3. Immunohistochemical Method

p16INK4a Monoclonal Antibody (1E12E10) Thermo branded antibody was used. The antibody is ready for use. Dyeing was done manually. The painting method is as follows. Sections were taken from paraffin blocks on a 3-4 micron thick adhesive slide. Sections were kept in an oven at 56 degrees overnight. The next day, the sections were kept in three separate xylenes for 5 minutes. Then, they were kept in graded alcohols for 5 minutes and washed in distilled water for 1 minute. They were boiled in 10% citrate buffer

(Sigma-Aldrich, USA, Ph6.0) solution for 10 minutes. The vessel's lid containing the boiled slides was opened and kept at room temperature for 20 minutes. The sections were rinsed with distilled water and kept in 10% hydrogen peroxide solution for 10 minutes, then washed again in distilled water and kept in a W block (ThermoScientific, USA) for 5 minutes. At the end of the period, the primary antibodies (ready for use) were dropped by shaking the W block on the sections without washing. Antibodies were incubated for 60 minutes. After incubation, washing was done in distilled water for 10 minutes. Then, it was passed to the secondary antibody stage and kept in biotin (ThermoScientific, USA) solution for 20 minutes, washed in distilled water for 5 minutes, and kept in streptavidin (ThermoScientific, USA) solution for 20 minutes. After washing in distilled water for 5 minutes, it was incubated in DAB chromogen (ThermoScientific, USA) for 7 minutes and washed. Finally, after 5 minutes of staining in Mayer's hematoxylin (Bio-Optica, Italy), it was passed through alcohol and xylene and closed with a mount.

2.4. Immunohistochemical Evaluation

Nuclear and cytoplasmic staining was considered positive in the immunohistochemical evaluation of pathological sections for p16 (INK4a). Cases without staining were considered negative. Cases with immunoreactivity below 10% were classified as 1(+), indicating low staining. Those with less than 25% immunoreactivity were categorized as 2(+), representing moderate staining. Cases with less than 50% immunoreactivity were deemed 3(+), indicating a high degree of staining.

2.5. Statistical analysis

The data obtained was transferred to IBM SPSS 26.0 software to create a dataset. Data distributions were examined using the Kolmogorov-Smirnov normality test. Independent variables were evaluated using the Mann-Kruskal Wallis test. Frequency distributions were evaluated with the Chi-Square goodness-of-fit test, and Pearson's Chi-Square test was used for comparisons in multi-dimensional tables. A p-value of <0.05 was considered significant in all statistical analyses.

3. Results

3.1. Statistical Results

When the frequency distribution of the ages of the 65 patients included in the study was examined, it was observed that the data were slightly skewed to the right. Still, it exhibited a normal distribution (p<0.05) (Figure 1.).

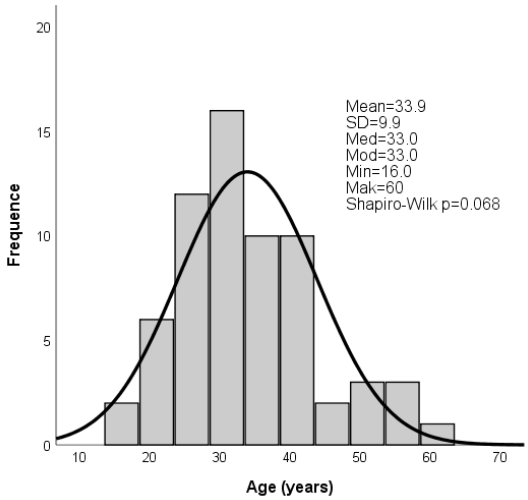

Figure 1. Frequency of age distribution among patients.

When the frequency distributions of the patients included in the study were examined according to the parameters of P16 immunohistochemical staining and pathological diagnosis, it was determined that the frequency distributions of both parameters showed statistical differences. Among the sampled patients, 50.8% were classified as having endometrial hyperplasia without atypia, while only 3.1% were diagnosed with AH / EIN. Regarding P16 immunohistochemical staining, it was found that the majority of patients, 46.2%, exhibited moderate P16 staining, 10.8% had high P16 staining, and 21.5% showed no expression of the P16 protein.

The statistical analysis showed that the age distribution of the patients included in the study did not adhere to a normal distribution when evaluated according to P16 immunohistochemical staining and pathological diagnosis groups ($p<0.05$). Therefore, the ages of the patients included in the study were assessed, taking into account P16 immunohistochemical staining and pathological diagnosis groups, using the Kruskal Wallis test (Table 1).

Table 1. Comparative Analysis of Age Distribution by Pathological Diagnoses and p16 Immunohistochemical Staining Intensity.

		Age				
		Mean±SD	**Min**	**Med**	**Max**	**P**
Pathological Diagnosis	**Proliferative endometrium**	30.5±10.8	18.0	29.0	50.0	0.160**
	Endometrial Hyperplasia without Atypia	35.8±10.2	21.0	33.0	60.0	
	AH / EIN	41.5±0.7	41.0	41.5	42.0	
	Endometrial Polyp	33.0±5.6	26.0	32.5	42.0	
P16 Immunohistochemical Staining	**Absent**	29.7±10.6	18.0	29.0	50.0	0.267**
	Low	38.0±12.7	19.0	36.0	60.0	
	Intermediate	34.4±8.2	21.0	33.0	57.0	
	High	32.3±6.9	24.0	33.0	42.0	

***Kruskal Wallis*

When the ages of the patients included in the study were examined based on the parameters of P16 immunohistochemical staining and pathological diagnosis, there was no statistically significant difference in age distributions (Table 1).

When the frequency distributions of patients included in the study were examined using a multidimensional table based on the parameters of P16 immunohistochemical staining and pathological diagnosis, a statistically significant difference was found at the p=0.000 level (Table 2).

Table 2. Variation in p16 Immunohistochemical Staining Across Diverse Pathological Diagnoses.

		P16 Immunohistochemical Staining (*n-%total*)				
		Absent	**Low**	**Intermediate**	**High**	**P**
Pathological Diagnosis	**Proliferative Endometrium**	14% (70.0)	6% (30.0)	0% (0.0)	0% (0.0)	***0.000*****
	Endometrial Hyperplasia without Atypia	0% (0.0)	6% (18.2)	24 % (72.7)	3 % (9.1)	
	AH / EIN	0 % (0.0)	0 (0.0)	1% (50.0)	1% (50.0)	
	Endometrial Polyp	0% (0.0)	2% (20.0)	5% (50.0)	3 % (30.0)	

****Pearson Chi-Square*

Notably, among patients classified as having proliferative endometrium, 70.0% showed no expression of the P16 protein, while 30% exhibited low expression. In contrast, among patients diagnosed with endometrial hyperplasia without atypia, 72.7% had moderate P16 protein expression, 18.2% had low expression, and 9.1% had high expression. It was observed that the number of patients diagnosed with AH / EIN was very low in the sample population. Among patients diagnosed with endometrial polyps, 50.0% exhibited moderate P16 protein expression, 20.0% had low expression, and 30.0% had high expression.

3.2. Histopathological and Immunohistochemical Findings

Histopathologically, the proliferative endometrium appears focally situated and irregularly shaped, with enlarged glands interspersed among normal endometrial glands. The gland-to-stroma ratio is generally between 1:1 and 2:1 (Figure II/A). Immunohistochemical staining with P16 revealed no immunoreactivity in endometrial stromal cells. The endometrial glandular epithelial cells exhibited variable and irregular immunoreactivity (Figure II/B-C-D).

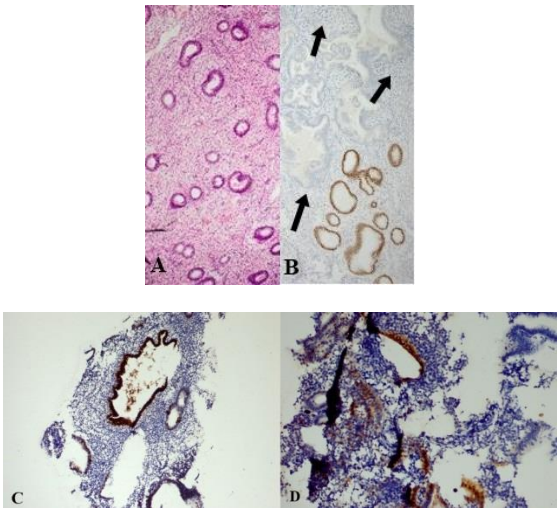

Figure 2. A: Proliferative endometrium. B: Areas indicated by the black arrow show no p16 immunoreactivity in endometrial stromal cells. Brown areas that are immunoreactive exhibit positive glandular staining. C and D: While there is no immunoreactivity in stromal cells, glandular cells exhibit varying degrees of immunoreactivity.

When examining the histopathological characteristics of endometrial polyps, they contain areas of simple and complex hyperplasia within a dense fibrous stroma. The endometrial glands are dilated and lined by a single layer of cells, typically covered with flattened epithelium, and lack mitotic activity (Figure III/ A-B). P16 immunoreactivities show weak to moderate immunoreactivity in stromal and glandular components (Figure III/ C-D-E-F-G-H). The histopathological findings of endometrial hyperplasia without atypia include cystic, expanded glands with occasional branching embedded within a cell-rich stroma. The lining cells are pseudostratified and columnar, with no cytological atypia and variable mitotic activity. Unlike proliferative endometrium, the stromal cells are denser, and clusters of glands exceeding the stromal volume are observed in endometrial epithelial cells, cytologically different from normal cells (Figure IV/ A). P16 immunoreactivity shows irregular, varying, weak to moderate reactions in stromal and glandular cells (Figure IV/B, C). In the histopathology of AH/EIN, there is an increased number of back-to-back glands that contain irregular branching with cellular atypia. The stroma is reduced due to crowded glands.

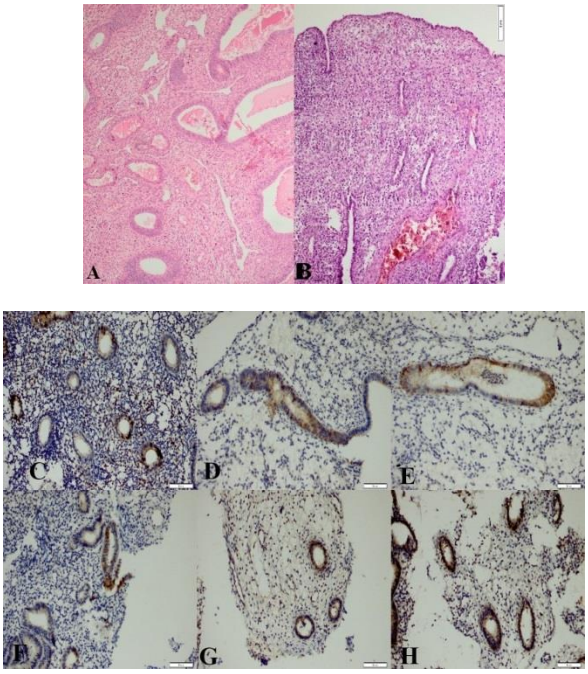

Figure 3. A, B: Endometrial polyps are characterized by their thick-walled vessels and the presence of complex hyperplastic areas. C, D, E, F, G, H: p16 immunoreactivity in various cases of endometrial polyps.

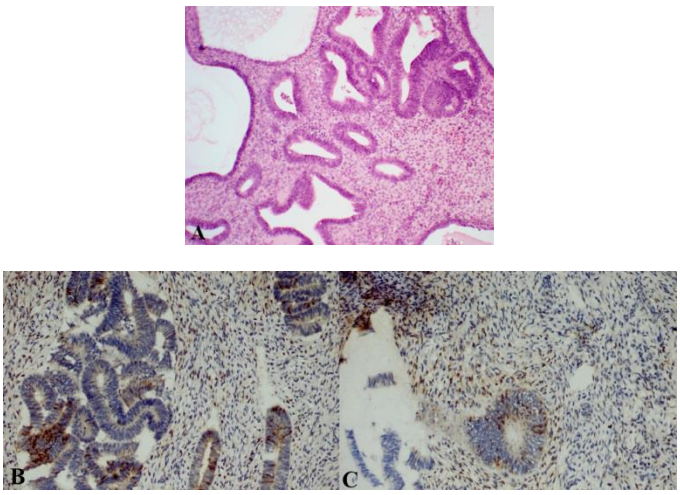

Figure 4 A: Atypical endometrial hyperplasias are characterized by a cell-rich stroma. Clusters of glands exceed the stromal volume in the glandular space, without cytological atypia. B, C: In atypical endometrial hyperplasias, stromal P16 immunoreactivity ranges from weak to moderate staining in glandular stromal areas, similar to what is observed in endometrial polyps.

Cellular atypia is classified as mild, moderate, and severe (Figure V/ A, B). P16 immunoreactivity showed intense expression, particularly in glandular areas (Figure V/ C, D, E, F).

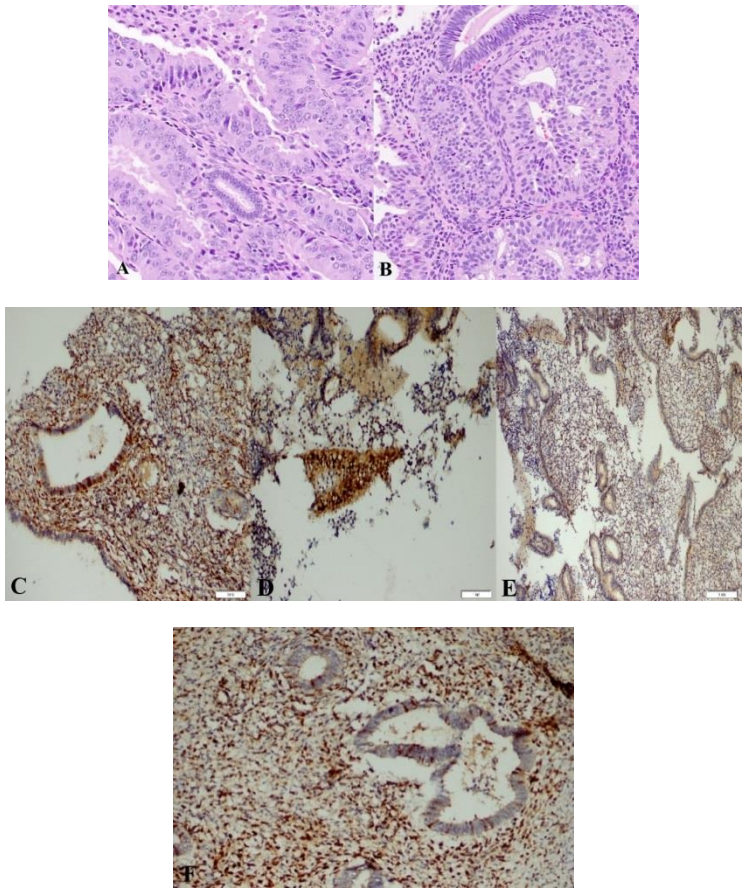

Figure 5. A, B: In AH/EIN, there is an increased number of glands arranged in a back-to-back configuration. Cellular atypia is also present. C, D, E, F: P16 expression shows intense immunoreactivity.

4. Discussion

Endometrial cancer is the most commonly diagnosed cancer of the female genital system in developed countries (16). Endometrial hyperplasias develop due to prolonged exposure of the endometrium to unopposed estrogen (17). Endometrial hyperplasia can progress to endometrial cancer if left untreated (18). Numerous physiological mechanisms have been identified transforming from endometrial hyperplasia to a malignant phenotype (19).

P16 is one of the most commonly used markers in gynecopathology cases, especially in cervical biopsies, displaying cytoplasmic immunoreactivity in HPV-associated lesions (20). Recent studies have utilized the P16 marker as a sensitive and distinctive indicator for endometrial serous adenocarcinoma and endometrial intraepithelial carcinoma (21). While numerous studies have been conducted on the cervix, more research is needed on the endometrium (22). P16 is known to be one of the tumor suppressor proteins with antiproliferative effects in tumor development. Studies have reported increased P16 expression with aging, oxidative stress, and DNA damage (23). In the literature on endometrial cancer, increased expressions of P16 have been reported (24).

Research has shown that P16 expression is rarely observed in normal endometrial stromal and glandular cells. However, the intensity of

staining varies in simple atypical and endometrial polyps. Reviewing the literature regarding the risk of malignancy in endometrial polyps, the rates of endometrial carcinoma and endometrial hyperplasia vary among studies (25). A significant reason for this variation may be the inability to standardize the diagnostic methods used for endometrial polyps and the inclusion of patients who were incidentally diagnosed with endometrial polyps through endometrial curettage, along with patients who strongly suggested endometrial polyps in preoperative evaluation (26). Although endometrial polyps are considered benign stromal neoplasms, data supporting this hypothesis are limited (27). Some studies have mentioned a clonal 6p21 gene in polyps limited to the endometrial mesenchymal component (13, 28). Moreover, amplification of the HMGIC gene has been found in endometrial polyps, and nuclear HMGIC gene expression has been identified in stromal cells of endometrial polyps. The HMGIC gene is known to be expressed benignly and is rarely found in mesenchymal and malignant tumors (29).

In our study, patients diagnosed with endometrial polyps exhibited varying levels of P16 protein expression, with 50.0% showing moderate expression, 20.0% displaying low expression, and 30.0% having high expression. The mechanism and significance of P16 expression in non-neoplastic gynecological lesions still require a comprehensive understanding, and it remains uncertain whether stromal proliferation is clonal in endometrial polyps. The mechanism and significance of P16 expression in non-neoplastic gynecological lesions have yet to be fully understood (30).

In the study conducted by Onat and colleagues (31), the subjects were categorized into the following groups based on their endometrial conditions: atrophic endometrium, endometritis, proliferative endometrium, secretory endometrium, decidualization, endometrium with irregular proliferation, endometrial polyp, endometrial hyperplasia, and endometrial adenocarcinoma. Subsequently, the patients were classified according to their age groups: <40 years as Group 1, 40-54 years as Group 2, and >55 years as Group 3.In the study that included 2023 patients, the mean age of the participants was 47.1 ± 10.0. The results for irregular proliferative endometrium, proliferative endometrium, secretory endometrium, decidualization, insufficient material, and endometrial hyperplasia without atypia showed significant differences across age groups. Their findings show that atypical results can also be observed in patients under 40. Our study found no significant difference due to the small number of cases. However, hyperplasia was also observed in patients in the younger age group. Patients diagnosed with endometrial hyperplasia without atypia showed varying P16 protein expression (32). The number of patients diagnosed with atypical endometrial hyperplasia was very low in the sample population.

In their study, Yoon and colleagues observed weak to moderate P16 expression in precancerous lesions and intense P16 expression in cases of AH/EIN and endometrial cancer (33). Similarly, Matson and colleagues observed intense P16 immunoreactivity in 63 cases of serous endometrial carcinoma and found moderate to intense expression varying in AH/EIN cases (34). Stewart and colleagues, in their study, examined P16 immunoreactivity in endometrial stromal cell expression. They found intense P16 expression in stromal cells in 32 cases of endometrial polyps (35). Our study also found results that support these three studies. P16 expression increases from normal endometrium to hyperplasia. While 3(+) positive staining was never observed in the proliferative endometrium group, it was predominantly seen in the AH/EIN group. Additionally, weak to moderate stromal and glandular stainings were observed in endometrial polyps and endometrial hyperplasia without atypia.

As the incidence of endometrial cancer increases, early diagnosis becomes crucial for effective treatment (36). Therefore, various factors that play a role in carcinogenesis are being investigated. Some endometrial hyperplasias are known to be precursors of endometrial cancer. In our study, P16 immunohistochemical expression was absent in normal endometrium, higher in atypical

endometrial hyperplasias than endometrial hyperplasia without atypia ones, and increased in endometrial polyps.

5. Conclusion

In conclusion, our study conclusively demonstrates that P16 expression shows a progressive increase from normal endometrium, through endometrial hyperplasia without atypia, to atypical hyperplasia/endometrioid intraepithelial neoplasia (AH/EIN). While endometrial polyps are generally benign, their occasional association with cancer risks, especially in postmenopausal women presenting with vaginal bleeding, underscores the need for vigilance.

Although endometrial hyperplasias are more commonly linked to precancerous states, the potential malignancy risk in endometrial polyps should not be disregarded. Effectively managing these conditions necessitates a personalized approach, considering age, menopausal status, symptoms, and other clinical risk indicators.

Notably, stromal P16 expression, while not conclusively indicative of a precancerous condition, can serve as a critical marker in distinguishing endometrial polyps. This marker merits thorough consideration in the overall assessment of cancer risk. Moreover, P16-induced cellular senescence in various benign mesenchymal neoplasms suggests that P16 staining in biopsy and curettage tissues could be a valuable diagnostic tool.

Our findings highlight the importance of P16 as a biomarker in the clinical evaluation of endometrial pathologies and advocate for its integration into routine diagnostic protocols to enhance the accuracy of cancer risk assessments in women with endometrial disorders.

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Ethics

Ethics Committee Approval: The study was received ethical approval from the Faculty of Medicine Ethics Committee, Kafkas University, with reference number 10, on November 23, 2021.

Informed Consent: The authors declared they get consent from the patients.

Copyright Transfer Form: Copyright Transfer Form was signed by all authors.

Conflict of Interest: The authors declare that they have no competing interests.

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