Examination of EZH2 expression on gynecological histological samples

Doctoral (PhD) Thesis

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Abbreviations

AIS	Adenocarcinoma *in situ*
CI	Confidence interval
CIN	Cervical intraepithelial neoplasia
ECA	Endocervical adenocarcinoma
EMC	Endometrial carcinoma
EZH2	Enhancer of zeste homolog 2
EMT	Epithelial-mesenchymal transition
H&E	Hematoxylin and eosin
HPVA	Human papillomavirus-associated adenocarcinoma
HSIL	High grade squamous intraepithelial lesion
IECC	International Endocervical Adenocarcinoma Criteria and Classification
ISGyP	International Society for Gynecological Pathologists
(i-)SMILE/ISMC	(Invasive) stratified mucin-producing carcinoma
LSIL	Low grade squamous intraepithelial lesion
NHPVA/HPVI	Non-HPV-associated/HPV independent adenocarcinoma
NOS	Not otherwise specified
p53abn	Abnormal p53 immunostaining / TP53 mutation
Rb	Retinoblastoma
RMS	Rhabdomyosarcoma
TCGA	The Cancer Genome Atlas
UCS	Uterine carcinosarcoma
WHO	World Health Organization

I. INTRODUCTION

Gynecology is one of those specific areas of pathology where immunohistochemical examinations play a prominent role in the diagnosis of various conditions (reactive changes, benign or malignant tumors, and their precancerous states). In practical terms, it is not uncommon for the application of immunohistochemical panels to only succeed in distinguishing between different conditions.

Cervical cancer and endometrial cancer are among the most common malignant tumors of the female genital tract. Both types of tumors represent a heterogeneous group of diseases with different etiology, molecular background, response to treatment, and prognosis.

The occurrence of endocervical adenocarcinoma (ECA) is less common compared to squamous cell carcinoma (20-25%), but clinically it is characterized by a worse prognosis and histologically greater heterogeneity. Most invasive endocervical glandular carcinomas are associated with HPV (HPV-associated ECA). Unlike squamous cell carcinomas associated with high oncogenic risk HPV infection, where HPV plays a role, HPV does not play a role in the development of a significant percentage (10-15%) of glandular carcinomas, making this tumor group of outstanding clinical significance. The latter have a worse prognosis compared to HPVA adenocarcinomas.

In the 2020 classification of female genital tract tumors, the World Health Organization (WHO) categorizes cervical adenocarcinomas based on their etiology and clinical behavior into human papillomavirus-associated (HPVA) and HPV-independent (non-HPV-associated/HPV independent adenocarcinoma, NHPVA/HPVI) groups. The WHO largely accepted the 2018 International Endocervical Adenocarcinoma Criteria and Classification (IECC). HPVA ECAs were further classified based on the amount of identifiable mucus in the cytoplasm of tumor cells. We distinguish between usual types - including the villoglandular and micropapillary variants, and mucinous types - including the not otherwise specified (NOS), the intestinal (goblet cell), the signet ring cell, and the invasive stratified mucin-producing carcinoma (i-SMILE/ISMC) variants, of ECAs. New entities in the WHO 2020 classification include adenosquamous, mucoepidermoid, and adenoid basal cell carcinomas. NHPVA includes gastric type, clear cell, mesonephric, and endometrioid ECAs. Invasive adenocarcinoma NOS is a category applied to very rare malignant tumors that cannot be morphologically classified into any group.

Endometrial cancer is the most common form of invasive gynecologic malignancy. A rather aggressive and extremely rare subtype, uterine carcinosarcoma (UCS), accounting for <5% of uterine corpus tumors, previously classified as mixed epithelial and mesenchymal tumors (malignant mixed Müllerian tumor, MMMT), is currently classified as a separate type of endometrial carcinoma. This biphasic tumor consists of a malignant epithelial (carcinoma) and a malignant homologous or heterologous mesenchymal (sarcoma) component. The malignant epithelial component is usually high-grade carcinoma, most commonly serous carcinoma, followed by endometrioid, clear cell, undifferentiated, and mixed histotypes. The mesenchymal component is most commonly high-grade or undifferentiated sarcoma. Among the heterologous sarcomatous components, rhabdomyosarcoma (RMS) is the most common. However, chondrosarcoma, osteosarcoma, and liposarcoma can also be observed. Evidence supports that UCS is caused by an epithelial-mesenchymal transition (EMT), which allows carcinoma cells to transition into a mesenchymal/sarcomatous cell phenotype, enabling them

to migrate from the originating epithelial layer. Based on data from The Cancer Genome Atlas (TCGA), over 90% of UCS cases contain TP53 mutations, which can explain their aggressive clinical behavior.

Due to their histomorphological diversity, within individual gynecological tumors - as well as in the mentioned tumors - overlapping morphologies can be encountered, posing diagnostic challenges in everyday pathological practice when establishing an accurate diagnosis.

The EZH2 (Enhancer of zeste homolog 2) gene is one of the most studied histone methyltransferases, a member of the polycomb protein group, which is responsible for the development and progression of numerous human cancers. Its increased expression has been demonstrated not only in hematological malignancies (e.g., lymphomas) but also in various epithelial malignancies (nasopharynx, breast, lung, stomach, colon, liver, pancreas, thyroid, prostate, bladder, ovary, uterus, cervix). Its increased expression has been correlated with the aggressive clinical behavior of tumors. As a result, EZH2 can serve as both a diagnostic and prognostic marker in oncology.

The physiological role of EZH2 in relation to the female reproductive organs is not fully elucidated. Regarding gynecological tumors, increased expression of EZH2 has been described in cervical squamous cell carcinoma, endometrial, and ovarian tumors. However, we did not find literature data regarding EZH2 expression in cervical adenocarcinoma or in tumors of the endometrium, including carcinosarcoma.

II. AIMS

Our aim was to investigate the expression of EZH2 through immunohistochemistry in various gynecological tissue samples. Despite the diversity of gynecological tumors, there is limited literature available regarding the diagnostic applicability of EZH2. While EZH2 expression is known in cervical squamous cell carcinoma, we supplemented our immunohistochemical examinations in our institute with this marker. However, we found no data on EZH2 expression in cervical glandular tumors. During the examination of a double cervix tumor sample with the EZH2 marker, we observed diffuse positive staining in the neoplastic glands of both the in situ squamous cell carcinoma and the invasive endocervical adenocarcinoma. We assumed that the increased positivity would be detectable in other cases as well, hoping that EZH2 would facilitate the diagnosis of cervical adenocarcinoma. According to a further observation, EZH2 showed similarly strong staining in the cells of the particularly rare and aggressive uterine carcinosarcoma (UCS). We found no previous studies on this matter. Evidence suggests that EZH2 promotes EMT, so we hypothesized that increased EZH2 expression may also play a role in the pathogenesis of UCS. To address the above, we aimed to conduct the following investigations:

II.1. In the first part of the thesis, we aimed to detect the nuclear expression of EZH2 in invasive and in situ endocervical adenocarcinomas (ECA and AIS), comparing them with normal endocervical epithelium and non-neoplastic endocervical lesions. We compared and analyzed the expression patterns and evaluated the specificity and sensitivity of EZH2 as an assisting marker in the differential diagnosis.

II.2. In the second part of the study, we investigated the expression of EZH2, as well as the p16 and p53 biomarkers, in uterine carcinosarcomas, and analyzed their possible roles in the diagnosis and histogenesis of UCS components and histotypes.

III. SUBJECTS AND METHODS

III.1. Patients and specimen collection

Primarily from the archives of the Department of Pathology at the University of Pécs and partly from the Pathology Department of Balassa János Hospital in Tolna County (Szekszárd), we conducted retrospective studies on tissue samples prepared from formalin-fixed, paraffin-embedded blocks. These studies were conducted with the approval of the Regional Research Ethics Committee of the University of Pécs (PTE/57682/2017, KK/644-1/2020). The clinicopathological characteristics of the individuals involved - such as their age, the number of histological examinations performed, the timing, and the results - were recorded in an Excel spreadsheet format suitable for statistical analysis.

III.1.1. Immunohistochemical analysis of EZH2 expression in endocervical lesions

We included cases diagnosed between 2007 and 2017, which originated from tissues removed during biopsy, conization, and hysterectomy procedures.

We examined fifty-four neoplastic endocervical lesions, of which thirty-seven were invasive endocervical adenocarcinomas (ECA) and seventeen were in situ adenocarcinomas (AIS). Among these cases, twelve also showed high-grade squamous intraepithelial lesions (HSIL) concurrently. Only one sample showed concurrent low-grade squamous intraepithelial lesion (LSIL).

In total, we analyzed thirty-two non-neoplastic endocervical lesions (15 reactive atypia, 9 microglandular hyperplasia, 3 tuboendometrioid metaplasia, 3 tunnel cluster, 2 endometriosis).

We also reviewed adjacent normal endocervical glandular epithelium, which was present in 34 out of the 54 malignant cases and in all non-neoplastic samples.

III.1.2. Immunohistochemical analysis of EZH2, p16 and p53 expression in uterine carcinosarcomas

To study the expression of EZH2, p16, and p53 markers, we collected consecutive cases of uterine carcinosarcomas diagnosed between 2012 and 2019 from the archives of the Department of Pathology at the University of Pécs.

We performed immunohistochemical examinations on samples from a total of 28 uterine carcinosarcoma cases, including 22 from hysterectomies and 6 from biopsies.

III.2. Methods

III.2.1. Histochemistry

Formalin-fixed and paraffin-embedded tissue samples stained with hematoxylin and eosin (H&E) were available in all cases. These slides were re-evaluated to select the most appropriate representative blocks containing both the epithelial and mesenchymal components for immunohistochemical studies for each patient, particularly in the case of carcinosarcomas. Endocervical adenocarcinomas were categorized according to the International Endocervical Adenocarcinoma Criteria and Classification (IECC). The epithelial and mesenchymal components of carcinosarcomas were further classified into additional histotypes according to the current WHO classification. Histological patterns according to IECC and WHO criteria were detected on these original, routine H&E-stained slides.

III.2.2. Immunohistochemistry

Prior to immunohistochemistry, formalin-fixed paraffin-embedded tissue specimens were cut into 4-µm-thick sections and dried for 20 min at 60 °C.

Immunostaining was performed using Leica Bond Max autostainer (Leica Biosystems, Bannockburn, IL) and Leica Bond Polymer Refine Detection Kit (Leica Biosystems, Newcastle Upon Tyne, UK). The mouse monoclonal EZH2 antibody (clone 6A10) was obtained from Leica Biosystems (Newcastle Upon Tyne, UK) and used at a dilution of 1:200. The rabbit monoclonal p53 antibody (SP5 clone) was obtained from Thermo Fisher Scientific (USA) and was also used at a dilution of 1:200.

The immunostaining protocol included deparaffinization and pH 9 epitope retrieval for 20 min, peroxidase blocking for 5 min, primary antibody incubation for 15 min, post-primary rabbit anti-mouse IgG for 8 min, polymer anti-rabbit Poly-HRP-IgG for 8 min, diaminobenzidine chromogen for 10 min, and hematoxylin counterstain for 5 min. Positive and negative controls were included in all reactions.

The mouse monoclonal Cintec p16 antibody (E6H4 clone) was detected using the Ventana Medical Systems Inc. (Tucson, AZ, USA) ready-to-use (RTU) form on the Benchmark Ultra automated staining platform. The immunostaining protocol included fully automated deparaffinization, 64-minute antigen retrieval at pH 8 (at 97°C), 20-minute primary antibody incubation, and the use of the manufacturer's Ultraview DAB Kit.

III.2.3. Evaluation of immunoreactivity

In both parts forming the basis of this thesis, I conducted the evaluation of all immunoreactions, which were ultimately reviewed by my supervisor, Dr. Krisztina Kovács. These evaluations will be referenced under her name in all subsequent sections.

III.2.3.1. Immunohistochemical analysis of EZH2 expression in endocervical lesions

The semi-quantitative analysis of immunoreactions was independently performed by three board-certified pathologists (Endre Kálmán, Krisztina Kovács, Angéla Oszter), each with over 15 years of professional experience. Cases were considered positive if clear positive staining was visible at 40x magnification. Positive cases were further classified based on the percentage of cells with nuclear staining: <10% focal positive " + ", 10-50% partially positive " ++ ", and >50% diffuse positive " +++ ".

The analysis of immunoreactivity included not only neoplastic and non-neoplastic lesions but also adjacent normal glandular epithelium if present in the section. Concurrent squamous cervical intraepithelial neoplasia was not considered in the evaluation.

III.2.3.2. Immunohistochemical analysis of EZH2, p16 and p53 expression in uterine carcinosarcomas

In this study, the semi-quantitative determination of EZH2 and p16 immunoreactivity was separately performed by two board-certified pathologists (Krisztina Kovács, Angéla Oszter) for both the epithelial and mesenchymal components. Similar to the previous examination, cases showing clear positivity at 40x magnification were considered positive, which were further classified based on the percentage of tumor cells with nuclear staining (+: <10%, focal positive; ++: 10-50%, partially positive; +++: >50%, diffuse positive) and staining intensity (0: no staining, 1+: weak, 2+: moderate, 3+: strong). Consistent with previous studies, the staining index was calculated as the product of the percentage of stained cells and staining intensity on a scale from zero to nine. Based on this, tumors were categorized into high expression (staining index > 4) *vs.* low expression (staining index ≤ 4) categories. Similar to other studies, only brown color reaction observed in the nucleus was considered positive for both markers. Cytoplasmic staining of p16 was disregarded.

The p53 immunoreactivity was determined according to the recommendations of the International Society for Gynecological Pathologists (ISGyP) (experts: Krisztina Kovács, Klára Éles). The p53 staining pattern was classified as wild-type or aberrant (the latter further subclassified as diffuse nuclear, null, or cytoplasmic expression). Firstly, the nuclear staining pattern was examined to determine whether the distribution and intensity of staining corresponded to the criteria for diffuse nuclear p53 pattern (strong and diffuse nuclear staining in over 80% of cells) or null p53 pattern (complete absence of nuclear staining in all cells). If neither pattern was confirmed, the second step involved evaluating cytoplasmic staining to differentiate between p53 cytoplasmic pattern (ranging from moderate to strong cytoplasmic staining) and wild-type p53 pattern (scattered nuclear staining).

III.2.4. Statistical evaluation

III.2.4.1. Immunohistochemical analysis of EZH2 expression in endocervical lesions

The agreement between the evaluating pathologists was determined using the Intraclass Correlation Coefficient (ICC) for both malignant and benign lesions analyses. A two-way, absolute agreement type model was employed. Both individual and average reliability of

evaluations were calculated. The analysis was conducted using MedCalc statistical software (version 13.0.0.0, MedCalc Software bvba, Ostend, Belgium).

For further descriptive and analytical statistics, individual evaluations for lesions and, if present, normal glandular epithelium were converted into binary summed scores. Based on this, immunoreactivity was considered robust if at least two out of three experts gave ratings of "++" or "+++". Immunoreactivity was defined as negative/focally positive if a minimum of two expertsrated the case as "-" or "+". Normal glandular epithelium was included in the statistical analysis if it was present in the sampleaccording to at least two experts.

The summed evaluations of immunoreactivity for neoplastic (ECA and AIS) and non-neoplastic lesions were statistically compared using the Fisher's exact test (MedCalc). A "p" value below 0.05 was considered statistically significant.

The diagnostic performance of EZH2 overexpression (sensitivity, specificity, positive and negative predictive values) was also assessed using MedCalc during the following comparisons: a) neoplastic *vs.* non-neoplastic lesions, b) neoplastic *vs.* normal glandular epithelium, and c) neoplastic lesions *vs.* non-neoplastic lesions and normal endocervix.

III.2.4.2. Immunohistochemical analysis of EZH2, p16 and p53 expression in uterine carcinosarcomas

In this study, for EZH2 and p16, we calculated the average staining index scores for both components (low or high expression) by two evaluators. The values of staining percentage and intensity were aggregated for further analysis. Different p53 patterns were reassessed, and the final result was determined based on consensus. Staining indicesfor EZH2 and p16, as well as p53 staining patterns, were compared between epithelial *vs.* mesenchymal components, serous vs. endometrioid carcinoma components, and homologous *vs.* heterologous sarcoma components. Mixed and undifferentiated epithelial types were excluded from the histotype comparison due to their low case numbers. Depending on the sample size, we used the Chi-square test or Fisher's exact tests for comparing categorical values (staining index and p53 staining pattern), while the Mann-Whitney test was used for comparing ordinal values (staining percentage and intensity). Concordance between epithelial and mesenchymal components was evaluated using the kappa (K) test. All statistical tests were performed using the MedCalc program, and a "p" value less than 0.05 was considered statistically significant.

III.2.5. Photodocumentation

The sections were digitized using a 3D HISTECH Pannoramic MIDI scanner, and photodocumentation was carried out using Pannoramic and CaseViewer software.

IV. RESULTS

IV.1. Immunohistochemical analysis of EZH2 expression in endocervical lesions

During the reevaluation of the original H&E samples, a total of 54 endocervical adenocarcinoma cases were identified, comprising 37 invasive adenocarcinomas (ECA) and 17 *in situ* adenocarcinomas (AIS). Among these, 12 cases showed concurrent high-grade squamous intraepithelial lesion (HSIL), and one sample showed low-grade squamous intraepithelial lesion (LSIL). The average age of the patients was 44.5 years (ranging from 29 to 84 years).

According to the IECC classification, the vast majority (92%) of ECAs were classified as human papillomavirus-associated adenocarcinoma (HPVA). Within this group, the most common subtype was usual-type adenocarcinoma (88% of the cohort). This was followed by villoglandular, mucinous (not otherwise specified, NOS), mucinous intestinal, and finally, invasive stratified mucin-producing carcinoma (iSMILE) types (3%).

Only 3 ECAs (8%) were classified as non-human papillomavirus-associated adenocarcinomas (NHPVA), of which two were serous (papillary) and one was an endometrioid subtype.

All neoplastic endocervical lesions (ECA and AIS) were found to be EZH2-positive by all three evaluating pathologists. Out of these 54 cases, with one exception, all lesions (98.14%) showed increased EZH2 expression (robust overall score).

Out of the 54 malignant samples, adjacent normal glandular epithelium was observed in at least two evaluations in 34 cases (63%). On average, the three experts did not detect EZH2 immunostaining in 88.3% of the identified normal glandular epithelium cases, with focal positivity observed in only 11.7%. Immunohistochemical analysis resulted in a negative/focal positive overall score in all 34 cases.

A total of 32 non-neoplastic endocervical lesions (15 reactive atypia, 9 microglandular hyperplasia, 3 tuboendometrioid metaplasia, 3 tunnel clusters, 2 endometriosis) were analyzed. On average, 67.7% of the immunostain evaluations were negative, 24% were focally positive, and 8.3% were partially positive. In the overall assessments, 28 out of 32 cases (87.5%) were negative/focally positive, while the remaining 4 cases (12.5%) received a robustoverall score.

In all non-neoplastic samples, adjacent normal glandular epithelium was detected by all threeexperts. In these samples from normal endocervix, EZH2 was found to be negative in 95.84%, focally positive in 2.08%, and partially positive in the remaining 2.08% of cases. The overall assessment resulted in negative/focally positive outcomes in each sample.

The Fisher exact test showed a statistically significant difference (two-tailed $p < 0.0001$) between the overall scores of neoplastic vs. non-neoplastic lesions (increased overall score was observed in 53 out of 54 neoplastic lesions, compared to only 4 cases out of 32 non-neoplastic lesions).

The results of the diagnostic performance of EZH2 overexpression are as follows:

a) In distinguishing neoplastic lesions from non-neoplastic lesions, the sensitivity was 98.15% (95% CI = 90.11% - 99.95%), specificity was 87.5% (95% CI = 71.01% - 96.49%), positive predictive value was 92.98% (95% CI = 83% - 98.05%), and negative predictive value was 96.55% (95% CI = 82.24% - 99.91%).

b) In distinguishing neoplastic lesions from from all normal glandular epithelium samples (n=66), the average sensitivity was 98.15% (95% CI = 90.11% - 99.95%), specificity was 100% (95% CI = 94.4% - 100%), positive predictive value was 100% (95% CI = 93.28% - 100%), and negative predictive value was 98.46% (95% CI = 91.72% - 99.96%).

c) In distinguishing neoplastic from non-neoplastic lesions and normal endocervical epithelium samples combined (n=98), the sensitivity was 98.15% (95% CI = 90.11% - 99.95%), specificity was 95.88% (95% CI = 89.78% - 98.87%), positive predictive value was 92.98% (95% CI = 83% - 98.05%), and negative predictive value was 98.46% (95% CI = 94.17% - 99.97%).

In the analysis of immunoreactivity in neoplastic endocervical lesions (ECA and AIS), the ICC among evaluators was 0.53 for individual evaluations (95% confidence interval = 0.37 - 0.67), and 0.77 for average evaluations (95% confidence interval = 0.64 - 0.86).

In the immunohistochemical analysis of non-neoplastic endocervical lesions, the ICC was 0.8 for individual assessments (95% confidence interval = 0.68-0.89) and 0.92 for average assessments (95% confidence interval = 0.86-0.96).

IV.2. Immunohistochemical analysis of EZH2, p16 and p53 expression in uterine carcinosarcomas

In this study, 28 women diagnosed with uterine carcinosarcoma were included, with an average age of 70.5 years (ranging from 53 to 85 years). The formalin-fixed and paraffin-embedded tissue samples were obtained from 22 surgical (hysterectomy) resections and 6 biopsy (curette) samples. Histologically, the epithelial component consisted of 14 cases (50%) of serous carcinoma, 9 cases (32.1%) of endometrioid carcinoma, 2 cases (7.1%) of undifferentiated carcinoma, and 3 cases (10.7%) of mixed (serous and endometrioid) carcinoma. The mesenchymal component included heterologous elements in 7 cases (25%) and homologous elements in 21 cases (75%). Among the heterologous sarcoma components, 4 cases of chondrosarcoma and 3 cases of RMS were observed. The most common homologous component was endometrial stromal sarcoma (n = 14), followed by 3 cases of leiomyosarcoma, 3 cases of undifferentiated sarcoma, and 1 case of myxoid fibrosarcoma.

During immunohistochemical analysis, increased expression of EZH2 was slightly more common in the epithelial/carcinoma components (89.3%) compared to the mesenchymal/sarcoma components (78.6%). Similarly, increased p16 expression was slightly more common in the epithelial components (78.6%) compared to the mesenchymal components (62.5%). According to Fisher's exact test, there was no statistically significant difference in EZH2 and p16 expression between the epithelial and mesenchymal components (p=0.468 for EZH2, p=0.248 for p16). The sum of the nuclear staining percentage and intensity scores for EZH2 and p16 ranged from zero to six. Mann-Whitney tests showed no statistically

significant difference in the percentage of nuclear staining and intensity between the epithelial and mesenchymal components for both markers (percentage staining for EZH2: p = 0.074, p16: p = 0.076; intensity of staining for EZH2: p = 0.11, p16: p = 0.059).

Both serous and endometrioid histotypes showed noticeable staining with both markers. Highexpression of EZH2 was detected in almost all cases (89% in both endometrioid and serous carcinomas). Highp16 expression was also very frequently observed (in 88% of endometrioid carcinomas and 79% of serous cases). When comparing serous to endometrioid histotypes, no statistical differences were found in EZH2 and p16 staining scores (for serous *vs.* endometrioid histotypes: according to Fisher's exact test, the p-values for EZH2 and p16 staining index were 0.74 and 0.96, respectively; according to the Mann-Whitney test, the p-values for EZH2 and p16 staining percentage were 0.35 and 0.15, respectively, while for staining intensity, they were 0.37 and 0.26, respectively).

Both sarcoma subtypes similarly showed frequent high expression of EZH2 (79% in homologous components, 78.6% in heterologous elements) and p16 (60% in homologous cases, 72% in heterologous cases). No statistical difference was found between the two markers (between homologous and heterologous types: according to Fisher's exact test, the p-values for EZH2 and p16 staining index were 1 and 0.66, respectively; according to the Mann-Whitney test, the p-values for EZH2 and p16 staining percentage were 0.37 and 0.4, respectively, and for staining intensity, they were 0.1 and 0.48, respectively).

Aberrant p53 immunostaining was observed in 85.7% of epithelial components and 82.1% of mesenchymal elements, with statistically similar values (Fisher's test p = 1). Within the epithelial components, aberrant p53 expression was found in 66.6% of endometrioid carcinomas and 100% of serous carcinomas, which was statistically significant (Fisher's test p = 0.0474). Regarding the mesenchymal components, 76.2% of homologous sarcomas and all heterologous sarcomas (100%) showed aberrant expression (no statistical difference, Fisher's test p = 0.29). Aberrant diffuse nuclear p53 expression was observed in 81.2% of homologous sarcoma histotypes, while only 28.6% of heterologous cases exhibited this staining pattern (the difference was statistically significant, Fisher's test p = 0.032). Aberrant p53 null pattern was observed in 71.4% of heterologous sarcomas, while only 18.8% of homologous sarcomas showed this staining pattern (the difference was statistically significant, Fisher's test p = 0.0257). No other significant differences were found in p53 staining patterns among the components and various histotypes of UCS.

Moderate p53 cytoplasmic staining was observed only focally in 3 out of 28 cases, but none of the tumors showed aberrant cytoplasmic expression. In fact, in all three cases, diffuse nuclear p53 expression pattern was confirmed, overriding the cytoplasmic staining, thus attributing diffuse nuclear p53 IHC pattern to all three cases.

During concordance analysis, p53 showed agreement in 82.14% between epithelial and mesenchymal components (Cohen's Kappa: 0.34 = "fair agreement").

VI. NOVEL FINDINGS AND CONCLUSION

1. **According to the first aim (immunohistochemical analysis of EZH2 expression in ECA and AIS samples):**

- In this study, we observed that all cervical glandular neoplastic lesions showed positive EZH2 expression based on our results. Furthermore, we found the immunoreactivity to be very /diffuse. With one exception, all (98.14%) neoplastic lesions exhibited robust EZH2 expression.
- In contrast, this intense labeling of EZH2 was significantly less common (12.5% in 32 cases) in non-neoplastic glandular lesions (two-sided test $p < 0.0001$). EZH2 positivity was not found in any of the adjacent normal glands (0/66). Occasionally, false-positive staining was caused by squamous metaplasia or reserve cell hyperplasia.
- Moreover, increased EZH2 expression demonstrated excellent diagnostic test performance in distinguishing neoplastic lesions from non-neoplastic lesions and normal endocervical tissues. We calculated sensitivity of 98.15% and specificity of 95.88% in distinguishing neoplastic lesions from combined non-neoplastic lesions and normal endocervical tissue samples (n=98), with a positive predictive value of 92.98% and a negative predictive value of 98.46%. The average agreement among evaluators was excellent.
- Our data support the potential role of EZH2 in the pathogenesis of cervical glandular neoplasms.
- Based on our results, significant EZH2 protein expression is observed not only in invasive endocervical glandular carcinomas but also in their precursor lesions, suggesting that EZH2 appears early in the process of carcinogenesis.
- Since EZH2 expression was detected in all cases examined regardless of the presence of human papillomavirus (HPV), our results do not support the hypothesis that increased EZH2 expression in endocervical adenocarcinomas is due to the process mediated by HPV E7 oncoprotein.
- We assume that EZH2 is a significant and independent factor in endocervical carcinogenesis and its increased expression is likely a result of other context-dependent processes.
- Our data indicate that increased EZH2 expression has high diagnostic reliability, with sensitivity and specificity exceeding 95%. Therefore, EZH2 could be a reliable differential diagnostic marker for endocervical lesions.
- Furthermore, the detection of increased EZH2 expression could be a useful tool for differential diagnosis in diagnostically challenging small biopsy specimens or problematic endocervical lesions with uncertain margins due to mechanical/thermal damage in flat conization specimens in histological samples, and probably also in the recognition of lesion cells in cytological samples.

2. **According to the second aim (immunohistochemical analysis of EZH2, p16, and p53 expression in uterine carcinosarcomas):**

- During the study of uterine carcinosarcoma cases, we observed that EZH2 not only shows high expression in the epithelial component but also is similarly increased in the mesenchymal component.
- Based on our results, p16 is overexpressed similar to the EZH2 marker, and most UCS cases exhibit aberrant p53 expression. Additionally, we found pattern differences among UCS histotypes.
- Our data demonstrate the possible role of EZH2 in the pathogenesis of UCS, supporting the epithelial-mesenchymal transition (EMT) theory in humans.
- The increased expression of EZH2, p16, and aberrant p53 in the epithelial and mesenchymal components of UCS further supports the monoclonal theory of uterine carcinosarcoma tumorigenesis.
- According to our results, different p53 expression patterns exist between the serous and endometrioid histotypes of the epithelial component (aberrant *vs*. wild-type), as well as between the homologous and heterologous types of the mesenchymal component (aberrant/diffuse nuclear *vs.* aberrant/null pattern). These pattern differences may indicate different genetic characteristics and differentiation pathways, requiring further molecular pathological investigations.

VII. LIST OF PUBLICATIONS

VII.1. Publications related to the thesis

Makk E, Bálint L, Czifra J, Tornóczky T, Oszter A, Tóth A, Kálmán E, Kovács K.
Robust expression of EZH2 in endocervical neoplastic lesions.
Virchows Archiv 475(1): 95–104. (2019)

Q1 IF = 4.535 Number of independent citations: 8

Makk E, Bohonyi N, Oszter A, Éles K, Tornóczky T, Tóth A, Kálmán E, Kovács K.
Comparative Analysis of EZH2, p16 and p53 expression in uterine carcinosarcomas.
Pathology and Oncology Research 29:1611547 (2023)

Q2 IF = 2.8 Number of independent citations: 0

VII.2. Absracts thet can be cited related to the hesis

Makk E, Bálint L, Czifra J, Tornóczky T, Oszter A, Kálmán E, Kovács K.
Robust expression of EZH2 in endocervical neoplastic lesions.
Technology Transfer in Diagnostic Pathology 10th CE Regional Meeting

Gynecological Pathology. Visegrád, 2019. június 27-29.

VII.3. Other publications

Rideg O, Oszter A, **Makk E**, Kálmán E, Farkas K, Tornóczky T, Kovács K.
Wide Spectrum Analysis of Human Papillomavirus Genotypes in External Anogenital Warts.
Vaccines (Basel) 9(6):604. (2021)
Q1 IF = 4.82 Number of independent citations: 2

VII.4. Other abstracts and presentations

Makk E, Kovács K.
Divergent immunophenotypes in primary and recurrent granulosa cell ovarian tumors.
Young Pathologists Meeting. Budapest, October 14-15, 2016.

Makk E, Bálint L, Kálmán E, Kovács K.
Cold knife conizations during pregnancy: clinicopathology and consequences.
XVI. Cytology Congress. Siófok, March 30-April 1, 2017.

Makk E, Rostás T.
Radiopathological case reports.
Slovenian-Croatian-Hungarian-Slovakian Radiological Symposium.
Rogaska Slatina, Slovenia, 2018. március 15-17.

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