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# Approach to P16 and Ki-67 in the Cervical Intraepithelial Neoplasia staging

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***Abstract:*** *cervical cancer is a tumor resulting from infection with high-risk human papillomavirus and develops from precursors within the epithelium, which are now referred to as squamous intraepithelial lesions or cervical intraepithelial neoplasias (SIL/CIN). CIN is categorized into CIN1, CIN2, and CIN3 depending on the degree of epithelial involvement. The treatment of patients with pre-invasive cervical lesions relies on the determined histological classification. The primary objective of this study was to thoroughly assess the relevance of immunohistochemical staining for the biomarkers p16 and Ki67 in the evaluation of cervical squamous intraepithelial lesions (SIL). This research aimed to elucidate the relationship between the levels of expression of these biomarkers and the various stages of cervical intraepithelial neoplasia (CIN). By analyzing the correlation between p16 and Ki67 expression and the severity of CIN lesions, the study seeks to enhance the understanding of these immunohistochemical markers' diagnostic and prognostic value in clinical practices for managing HPV-related cervical pathology. Materials and methods. We analyzed cases of women with suspected cervical neoplasia during cytology investigation. H&E-stained and IHC slides of all biopsy samples were reviewed and classified according to the criteria outlined by the LAST project. All histological samples were processed according to routine procedures. Results. We found, that p16 expression is divided into three groups: negative, strong and diffuse block-positive, and focal or patchy positive reaction. In cases histologically classified as CIN1, p16 showed inconsistent positivity with a moderate intensity. The glandular epithelium displayed clear signs of atypia, and p16 revealed inconsistent moderate positivity. Ki-67 was expressed in cells nuclear in the basal and parabasal layers of the squamous epithelium in all CIN stages. The results showed significant p16 and Ki67 expression differences among different groups of CIN. The expression levels of P16 and Ki-67 showed a positive correlation with the severity of cervical lesions and are very helpful for distinguishing CIN1 from CIN2 and CIN3. Conclusions. The expression levels indicating a consistent increase in expression that reflects the progression. Utilizing both P16 and Ki-67 can help identify patients at a higher risk for Squamous Cell Carcinoma (SCC). The applications of p16 and Ki-67 have proven to be important supplementary tools in evaluating the actual characteristics of these lesions. The staining results of p16 and Ki-67 across various histological grades of cervical lesions underscore their usefulness in validating histological diagnoses.*

**Keywords.** Cervix Neoplasms; Immunohistochemistry; Ki-67 Antigen; Cancer of the Uterine Cervix; Squamous Cell; p16 Protein.

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## Introduction

Cervical cancer is the fourth most common cause of cancer-related deaths among women [1]. Current clinical data reveal that the occurrence of cervical cancer among younger women is steadily rising each year. Approximately 99.7% of cervical cancer cases are linked to Human Papilloma Virus (HPV), especially high-risk HPV (HR-HPV) [2]. Today's cervical cancer prevention involves detecting high-risk HPV genotypes and precancerous lesions, followed by management that can include monitoring, repeat screenings, colposcopy, and possible treatment [3]. Like many types of cancer, it begins as a clearly recognizable and manageable pre-malignant state, which is influenced by a significant known factor called persistent high-risk human papillomavirus (hrHPV) infection, allowing for prevention [4].

Cervical squamous intraepithelial lesions (CSIL) indicate a progression from normal cervical tissue to squamous carcinoma (SCC). About 80% of low-grade lesions (LSIL-CIN1) resolve on their own within one to two years. High-grade lesions (HSIL-CIN2-3) require biopsy and removal to prevent progression, while LSIL/CIN1 management focuses on supportive care and monitoring, as 10% to 15% may progress to HSIL or CIN2-3 [5].

According to the LAST Project's 2012 classification, Cervical SIL is divided into low-grade (LSIL) and high-grade (HSIL) squamous intraepithelial lesions. LSIL corresponds to CIN1, indicating a non-carcinogenic HPV infection that often resolves on its own. HSIL, equivalent to CIN2/3, is a precancerous lesion that typically requires surgical intervention to prevent progression to cervical cancer. The standard practice is to treat CIN3 and most CIN2 lesions through macrocyclic or conical resection of the cervical transformation zone. Spontaneous regression occurs in approximately 40–50% of CIN2 and about 30% of CIN3 lesions [5,6]. As a result, removing all high-grade cervical lesions (CIN2/3) might result in overtreatment. Clinically, conditions like cervicitis or CIN1, identified through a colposcopy biopsy, have the potential to advance to cervical cancer quickly without first transitioning through CIN2 and CIN3 stages. In such cases, cervical cancer could arise during standard follow-up [7]. Creating a detection technique to quickly and accurately distinguish between LSIL and HSIL is essential for effective patient treatment. Immunohistochemistry assesses biochemical changes in tissues, supporting diagnoses and providing insights into disease progression and treatment outcomes [8].

P16, a protein that acts as a tumor suppressor, prevents the activation of CPK and oversees cell cycle and proliferation through the phosphorylation of PRB. The immunohistochemical presence of p16 might reduce PRB activity as part of a negative feedback mechanism [5]. Persistent HPV infection causes overexpression of P16, which is important for cervical cancer screening but may not be sufficient for diagnosis alone. Ki-67, a nuclear antigen indicating cell proliferation, is rarely co-expressed with P16 in normal tissues [3]. The assessment of dual expression is essential for evaluating the risk of cervical cancer, as the occurrence of these markers is mutually exclusive in healthy cervical cells [9].

## Aim

We carried out this research to investigate the utility of the immunohistochemical staining for p16INK4a and Ki-67 in the pathological assessment of cervical tissue samples from the cervix uteri and represent the correlation between reaction expressiveness and stages of CIN.

## Materials and methods

We analyzed 30 cases from the pathology department of the clinical laboratory and divided them into 3 groups (CIN1, CIN2 and CIN3). The study included women under 50, who cytologically had LSIL (CIN1) and HSIL (CIN2/3) according to the Bethesda system for reporting cervical cytology. Patients had no previous treatment. Tissue samples were collected by colposcopic (punch) biopsy, cone biopsy, and endocervical curettage (ECC). All histological samples were processed according to routine procedures. All slides were investigated by four pathologists. Nuclear staining with P16 was evaluated according to four parameters: intensity, extent, continuity, and location, following the LAST criteria. Lesion extent was classified as diffuse (more than 50% of the epithelium) or focal (less than 50%). Location

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was determined by the presence of positive cells in the lower one-third, two-thirds, or full thickness of the epithelium for Ki-67. Lesions were categorized as strong and diffuse block-positive, negative, or focal/patchy positive for p16. Strong and diffuse block-positive lesions met LAST criteria, showing strong immunoreactivity from the basal layers, involving over one-third of the epithelium with continuous involvement. Negative immunostaining was defined by a lack of staining or weak, focal, and/or discontinuous staining. Nuclear staining by Ki-67 was deemed positive.

**Results**

Each of the three groups into which the cases were divided contained 10 cases of CIN1, CIN2 and CIN3, respectively. The average age for the CIN1 group was 32.7± 9.2 years, CIN2-36.1± 6.7 and CIN3-40.1± 6.4. The women had no history of pregnancy, previous invasive interventions or cancer. CIN1 group: 30%, 50%, and 20% showed strong and diffuse block-positive, focal or patchy positive, and negative expression for p16, respectively. Ki-67 expression in 1/3, 2/3 and 3/3 epithelium thickness were 70%, 20% and 10% positive nuclear reaction, respectively. CIN2 group: 80%, 20%, and 0% showed strong and diffuse block-positive focal, patchy positive, and negative expression for p16, respectively. Ki-67 expression in 1/3, 2/3 and 3/3 epithelium thickness were 50%, 40% and 10% positive nuclear reaction. CIN3 group: 90%, 10%, and 0% showed strong and diffuse block-positive, focal or patchy positive, and negative expression for p16, respectively. Ki-67 expression in 1/3, 2/3 and 3/3 epithelium thickness were 10%, 0% and 90% positive nuclear reaction (Figure 1).

**Discussion**

Globally, cervical cancer is the fourth most common type of cancer in women, trailing behind breast, colorectal, and lung cancers, with 600,000 new diagnoses and 340,000 fatalities annually [5].

In the current research, 56,6% of HSIL (CIN2/3) cases exhibited positive block p16 expression and 10% focal or patchy p16 expression, while 0% exhibited negative p16 expression. Expression of Ki-67 was observed in 100%, in 30% of HSIL (CIN2/3) cases the reaction was considered in 1/3 of epithelium, 20% of cases had expression in 2/3 of epithelium thickness and 50% in cases with reaction in 3/3 of thickness. These results were comparable to those of Mehdi, Hajra K. et al, 2023 [2].

The study by Catalán-Castorena, O. et al. (2024) indicates a relationship between neoplasia development and HPV infection in various locations, particularly HPV types that increase the possibility of developing squamous intraepithelial lesions (SIL) [10].

Nicolas Wentzensen et al. cross-sectional study indicates that there is 100% sensitivity for CIN3 in older women with lesions linked to HPV16, which implies that the colposcopic assessment for p16/Ki-67–negative women can be postponed without risk [11]. Rama Kumari et al. found that P16 IHC is 100% sensitive, 85.71% specific and 95.59% accurate and Ki-67 IHC is 96.49% sensitive, 92.31% specific and 95.71% accurate in predicting positive results among cervical lesions. The agreement of P-16 HIC, Ki-67 and histopathological diagnosis is significant (P-value<0.00001) [12]. According to a study by Maryam Sadat Hosseini, Maryam Talayeh et al. (2023) the specificity and sensitivity of p16 are 100% and 58.73% respectively. The specificity and sensitivity of Ki-67 (Full thickness) are 100% and 60.32% respectively [6].

In clinical settings, p16 immunohistochemical staining helps differentiate between similar cervical lesions (CIN1 or CIN2), but previous research shows interpretation issues in certain cases. This highlights the need for new immune markers or diagnostic methods. Some studies indicate no significant differences in CIN grade distribution ($p = 0.765$), with similar abnormal group percentages across CIN1-3. Patients with diffuse, elevated p16INK4A and Ki67 levels faced a higher recurrence rate ($p = 0.024$) and a greater likelihood of progression to invasive cancer ($p = 0.016$) [15].

This study presents several limitations worth noting. Primarily, the relatively small sample size may undermine the external validity of the findings, potentially limiting their applicability to broader populations. Immunohistochemistry of p16/Ki-67 system is based on the subjective assessment of distribution of IHC staining

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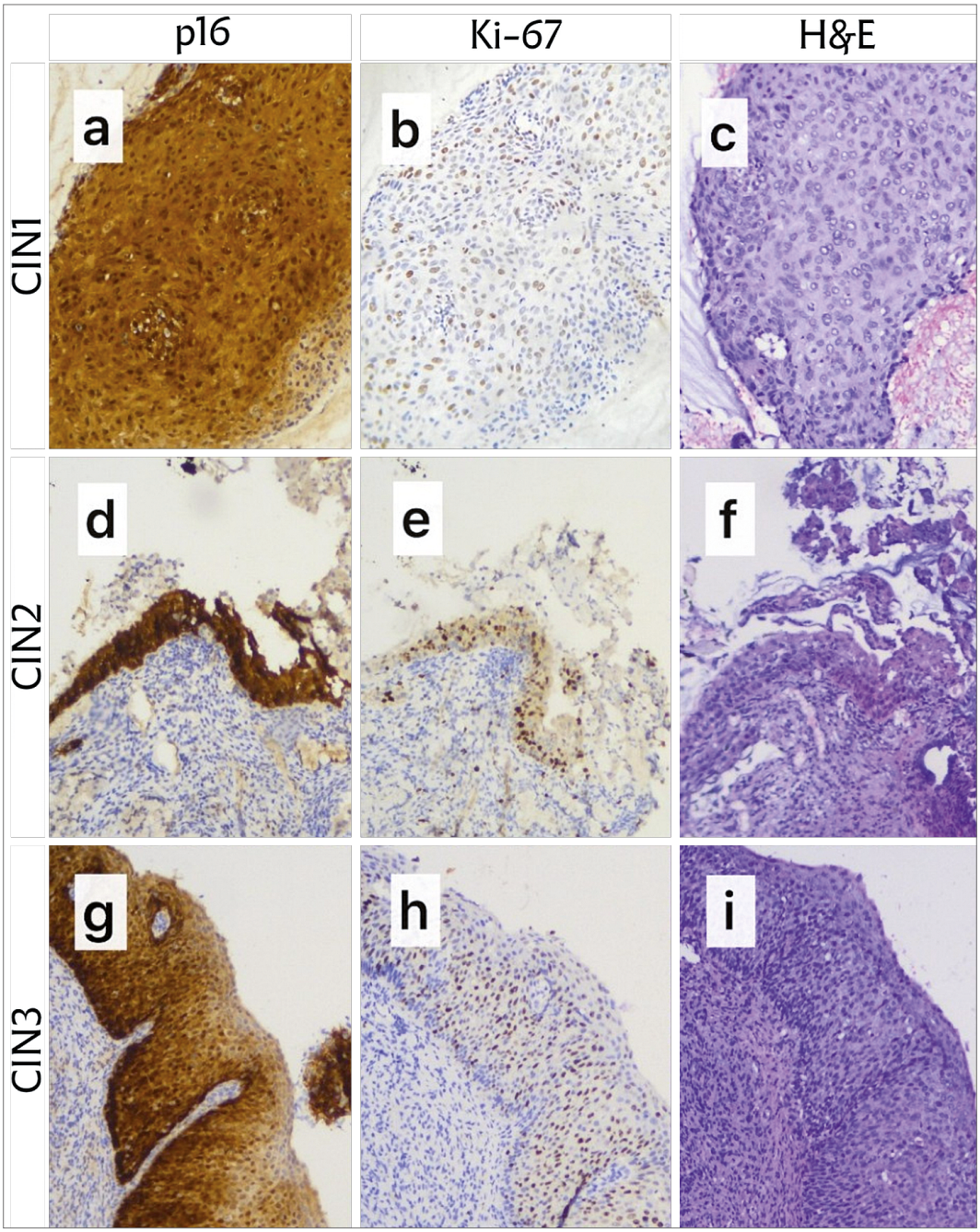


**Figure 1.** Expression of Ki-67 and p16 proteins in LSIL, HSIL.
**(a–c)** LSIL/CIN1 lesion: (**a**) p16 stain; (**b**) Ki-67 stain; (**c**) Hematoxylin and eosin (H&E) stain.
**(d–f)** HSIL/CIN2 lesion: (**d**) p16 stain; (**e**) Ki-67 stain;
**(f)** Hematoxylin and eosin (H&E) stain. (**g–i**) HSIL/CIN3 lesion: (**g**) p16 stain; (**h**) Ki-67 stain;
**(i)** Hematoxylin and eosin (H&E) stain

in CIN. The results of our study are similar to those of our colleagues with a larger number of patients, but there is no guarantee that the sample was representative. The absence of follow-up cases restricts the capacity to evaluate long-term outcomes. Long-term follow-up of patients could allow for more specific confirmation of the medical diagnosis, taking into account the further course of the neoplastic process–an example of a case of CIN3 progression to SCC. The cross-sectional design employed means that data collection occurred at a singular time point, thus precluding any longitudinal analysis. Additionally, the investigation was limited to only

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two biomarkers, which may constrain the breadth and depth of the findings. Utilizing additional markers could have enhanced the prediction of other cervical cancer parameters.

In the current study, the expression levels of both markers displayed a statistically significant positive correlation from CIN1 to CIN3, indicating a consistent increase in expression that reflects the progression from normal cells to high-grade squamous intraepithelial lesions (CIN2/3), culminating in cancer cells marked by an enhanced expression of the p16 marker (which represents the integration of HPV viral genes with host genes, resulting in immortalized cells), along with a gradual rise in the expression of Ki-67 protein.

**Conclusions**

In summary, histopathology remains the primary method for diagnosing cervical lesions, but p16 and Ki-67 are valuable supplementary tools that help assess these lesions' characteristics. A positive p16 stain in low-grade dysplastic lesions may suggest a risk of aggressive behavior and progression, while Ki-67 levels indicate the growth dynamics of these lesions. Elevated Ki-67 suggests an active disease process, influencing treatment decisions and follow-up urgency.

Using Ki-67 alongside p16 is crucial for distinguishing dysplastic from non-dysplastic lesions, guiding clinical strategies on monitoring, surgical intervention, or further evaluations. This approach aims to enhance patient outcomes by preventing overtreatment in low-risk cases. Overall, integrating p16 and Ki-67 into clinical protocols refines diagnosis and optimizes patient management, leading to better health outcomes for those with cervical lesions.

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**Conflict of interests**

No conflict of interests.

**Consent to publication**

All authors have got consent to publication of this article.

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A – Research concept and design, B – Data collection and analysis, C – Responsibility for statistical analysis, D – Writing the article, E – Critical review, F – Final approval of the article

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# Підхід до визначення P16 та Ki-67 у стадіюванні цервікальної інтраепітеліальної неоплазії

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***Анотація:*** *рак шийки матки - це пухлина, що виникає внаслідок інфікування вірусом папіломи людини високого ризику і розвивається з попередників в епітелії, які зараз називають плоскими внутрішньоепітеліальними ураженнями або цервікальними внутрішньоепітеліальними неоплазіями (SIL/CIN). Залежно від ступеня ураження епітелію CIN поділяють на CIN1, CIN2 і CIN3. Лікування пацієнток з преінвазивними ураженнями шийки матки залежить від визначеної гістологічної класифікації. Основною метою цього дослідження була ретельна оцінка релевантності імуногістохімічного забарвлення на біомаркери p16 і Ki67 в оцінці плоскоклітинних інтраепітеліальних уражень шийки матки (SIL). Метою цього дослідження було з'ясувати взаємозв'язок між рівнями експресії цих біомаркерів та різними стадіями цервікальної інтраепітеліальної неоплазії (ЦІН). Аналізуючи кореляцію між експресією p16 і Ki67 та ступенем тяжкості ураження CIN, дослідження спрямоване на поглиблення розуміння діагностичної та прогностичної цінності цих імуногістохімічних маркерів у клінічній практиці при лікуванні патології шийки матки, пов'язаної з ВПЛ. Ми проаналізували випадки жінок з підозрою на цервікальну неоплазію при цитологічному дослідженні. Забарвлені за допомогою H&E та ІГХ мазки всіх біоптатів були переглянуті та класифіковані відповідно до критеріїв,*

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*викладених у проекті LAST. Всі гістологічні зразки були оброблені відповідно до рутинних процедур. Результати показали значні відмінності в експресії P16 і Ki67 між різними групами CIN. Рівні експресії P16 і Ki-67 показали позитивну кореляцію з тяжкістю ураження шийки матки і є дуже корисними для розрізнення CIN1 від CIN2 і CIN3. Отже, рівні експресії вказують на послідовне збільшення експресії, що відображає прогресування. Використання як P16, так і Ki-67 може допомогти виявити пацієнтів з підвищеним ризиком розвитку плоскоклітинного раку (ПКЛ). Застосування p16 і Ki-67 виявилося важливим додатковим інструментом в оцінці фактичних характеристик цих уражень. Результати фарбування p16 і Ki-67 в різних гістологічних класах уражень шийки матки підкреслюють їх корисність для підтвердження гістологічних діагнозів.*

**Ключові слова:** новоутворення шийки матки, імуногістохімія, антиген Ki-67, білок p16, рак шийки матки, плоскоклітинний тип.