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ORIGINAL ARTICLE

IMPLEMENTATION OF P16/KI67 DUAL STAINING CYTOLOGY FOR DETECTING CERVICAL DYSPLASIA

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Abstract:
Cervical cancer is the fourth most common cancer among women worldwide. Vaccination against oncogenic human papillomaviruses (HPV) and effective screening have made cervical cancer preventable. Current screening methods, including cytology, HPV testing, and a combination of both, have limitations, highlighting the need for additional markers to identify high-grade cervical lesions (CIN2+).
p16/Ki67 dual immunocytochemistry staining is a biomarker with high sensitivity and specificity for detecting CIN2+ lesions. Incorporating this biomarker in triage, alongside cytology and HPV testing, can help avoid unnecessary referrals for colposcopy and biopsy. This study, conducted at the University Clinic for Gynecology and Obstetrics in Skopje over a one-year period, involved 40 female patients aged 21 to 65 years, all of whom underwent HPV DNA testing, cytological testing (LB) and p16/Ki67 dual staining. The study found a significant association between High-Grade Squamous Intraepithelial Lesion (HSIL) and p16/Ki67 dual staining (p=0.012), while no significant association was observed between Low-Grade Squamous Intraepithelial Lesion (LSIL) and p16/Ki67 staining (p=1.0).

***Key Words**: cervical dysplasia*; *HPV; immunocytochemistry; p16/Ki67.*

Introduction

Cervical cancer is the fourth most common malignant tumor in women globally and remains a major public health challenge, particularly in low- and middle-income countries (1). In 2020, 604,000 women were diagnosed with cervical cancer, and 342,000 died from it (2). However, vaccination against high-risk human papillomaviruses (HPV) and effective screening have made the disease mostly preventable (3).
The World Health Organization (WHO) launched a global initiative in 2020 to eliminate cervical cancer, aiming to bring its incidence below 4 cases per 100,000 women annually in all countries (1,4). The WHO's 90–70–90 target to be achieved by 2030, is for 90% vaccination of girls by age 15, 70% screening of women with high-performance tests at least twice by age of 45, and 90% treatment of women diagnosed with cervical precancer or invasive cancer (1,4).
In the Republic of North Macedonia, cervical cancer continues to be a significant concern. The incidence was 7 per 100,000 women in 2020, with 68 deaths in 2019 (5). The disease is most commonly diagnosed in women aged 35 to 44 years, with a mean age of diagnosis at 50 years. Notably, over 20% of cases occur in women older than 65 years (6).

Cervical cancer primarily develops because of chronic infection with high-risk HPV types, especially HPV 16 and HPV 18, which are responsible for 70% of cervical cancers worldwide (7,8). While the most of HPV infections resolve within one to two years without developing cancer, chronic infections can cause precancerous lesions that, if untreated, may progress to invasive cancer (9). Risk factors for cervical cancer include the oncogenic potential of the HPV type, immune status, sexually transmitted infections, parity, early pregnancy, hormonal contraceptive use and smoking (10).
Chronic HPV infection contributes to carcinogenesis via the E6 and E7 proteins, which deregulate the cell cycle (11). Abnormal cervical cells typically take 15–20 years to develop into cancer, though in immunocompromised patients, e.g., untreated HIV infection, this will be sooner (10). Screening for cervical cancer has traditionally relied on cytology, either conventional (CC) or liquid-based (LBC). As the most of cervical cancers result from persistent high-risk HPV infection, many countries have implemented HPV DNA screening as the primary test (8). The WHO recommends HPV DNA testing as a primary screening method, with partial genotyping for HPV 16 and 18, cytology or colposcopy for triaging positive patients (12).
The WHO also recommends HPV DNA testing as the primary screening test for both the general female population and HIV-infected women (12). In places where HPV DNA screening is not yet feasible, the WHO suggests regular screening every 3 years using cytology or colposcopy as the primary test for both the general population and women living with HIV (5).
While cytology is very specific, it is not sensitive, resulting in large number of false-negative results (13,14). On the other hand, HPV DNA testing, is very sensitive (around 90%) but not specific, which can lead to unnecessary referrals to colposcopy or biopsy, particularly in younger women. Therefore, more effective triage markers are needed to identify women at higher risk for CIN2+ lesions despite normal cytology (15,16).
p16/Ki67 dual cytological staining is a promising triage test that is highly sensitive and specific for detecting high-grade cervical lesions (17).
p16 inhibits cyclin-dependent kinases and regulates the cell cycle, while Ki67 is a marker of cell proliferation (13,17). The co-expression of these proteins indicates cell cycle deregulation and can predict the development of high-grade lesions (13).
This study aims to explore the correlation between p16/Ki67 immunocytochemical status and cytologically verified squamous intraepithelial lesions.

Materials and Methods

This study was conducted over a one-year period and involved 40 patients, aged between 21 and 65 years. All the patients underwent HPV DNA testing with typing, cytological testing (Liquid-Based Cytology or LBC), and p16/Ki67 dual cytological staining. Only patients who tested positive for high-risk HPV types during screening were included in the analysis, regardless of whether they had cytologically confirmed lesions. The study took place at the University Clinic of Gynecology and Obstetrics in Skopje, and all cytological tests (LBC) and p16/Ki67 immunocytochemical staining were performed in the University Cytology Laboratory.
Liquid-Based Cytology (LBC) was used for sample collection in this study. In this method a sample is obtained using a brush, which is placed in a liquid medium. The cervical smears are classified according to the Bethesda system (2001).
For p16/Ki67 dual cytological staining, the CINtec® PLUS cytology kit was used to detect cells with neoplastic transformation by identifying the presence of both p16 and Ki67

proteins. A positive test result is indicated when at least one cervical epithelial cell shows brown cytoplasm and a red-stained nucleus.
HPV DNA testing was performed using real-time multiplex PCR assays. The extracted DNA samples undergo real-time PCR amplification using commercial kits that allow simultaneous detection and differentiation of DNA from 19 high-risk HPV types (hrHPV: 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 69, 73, 82) and 9 low-risk HPV types (lrHPV: 6, 11, 40, 42, 43, 44, 54, 61, 70), along with an internal control.
The participants were selected based on specific inclusion and exclusion criteria. The inclusion criteria were the following: patients aged 21 to 65 years, patients with cytologically confirmed low- or high-grade squamous intraepithelial lesions (LSIL or HSIL) who tested positive for high-risk HPV types via HPV DNA testing, and patients who tested positive for high-risk HPV (HR-HPV) DNA but had normal cytology results. The exclusion criteria included patients with low-risk HPV types and patients diagnosed with invasive cervical cancer on clinical examination, regardless of cytological findings. The patients were divided into three groups. One group consisted of patients who tested positive for HR-HPV DNA but negative for cytology. The second group consisted of patients who tested positive for HR-HPV DNA and had a cytological diagnosis of LSIL. The third group consisted of patients who tested positive for HR-HPV DNA and had a cytological diagnosis of HSIL

Results

Table 1. Total Number of p16/Ki67 Immunocytochemistry Positive Patients.

	Frequency of p16/Ki67 Immunocytochemistry	
Category	**No.**	**Percentage**
Positive	6	15%
Negative	34	85%

A total of 40 patients with high-risk HPV were included in the study, out of which 6 patients were positive for the p16/Ki67 dual cytological staining, while the remaining 34 were negative.

Table 2 & 3. Average Age of Patients.

No.	**Average Age**	**Minimum age**	**Maximum age**	**Std. Dev.**
40	34	21	65	11.03607

p16/Ki67	**No.**	**Average Age**	**Std. Dev.**
Positive	6	35.5000	15.65567
Negative	34	33.7353	10.31124

The average age of the patients included in the study is 34 years. The average age of patients who were positive for dual cytological staining is 35.5 years, while for the

negative patients, it is 33.7 years. The value of the Independent Samples Test T-test is 0.58 > p 0.05.

Table 4. Representation of Cytological Findings

Category	No.	Percentage
Low-Grade Squamous Intraepithelial Lesion (LSIL)	26	65%
High-Grade Squamous Intraepithelial Lesion (HSIL)	2	5%
Negative PAP Test	12	30%

Out of the 40 patients, who underwent LBC cytology, Low-Grade Squamous Intraepithelial Lesion (LSIL) was detected in 26 patients (65%) and High-Grade Squamous Intraepithelial Lesion (HSIL) was detected in 2 patients (5%).

Table 5. LSIL/HSIL - p16/Ki67 Co-distribution

LBC		Immunocytochemistry Positive for p16/Ki67	Immunocytochemistry Negative for p16/Ki67	Total
HSIL	**No.**	2	0	2
	%	33.33%	/	
LSIL	**No.**	4	22	26
	%	66.67%	64.71%	
Negative PAP test	**No.**	0	12	12
	%	/	35.29%	
Total		6	34	40

In the group of patients with LSIL obtained by LBC cytology, out of a total of 26 patients, 4 patients were positive and 22 were negative for the p16/Ki67 dual cytological staining. This subgroup accounts for 66.7% of the total number of positive patients for the dual staining. In the patients with LSIL, 15.4% were positive for the p16/Ki67 dual staining test. In the group of patients with HSIL obtained by LBC cytology, out of a total of 2 patients, both were positive for the p16/Ki67 dual cytological staining, which corresponds to 33.3% of the total number of patients who were positive for dual cytological staining. Among the patients positive for HSIL, 100% were also positive for the dual cytological staining test.

Discussion

The study included 40 patients who were positive for high-risk HPV. Among these, 6 (15%) were positive for the p16/Ki67 dual cytological staining, and 34 (85%) were

negative. The average age of patients was 34 years, with the positive group averaging 35.5 years and the negative group 33.7 years. However, the T-test result ($p = 0.58$) indicated no statistically significant age difference between the groups.
Cytological findings showed that LSIL was present in 65% of the patients, HSIL in 5%, and 30% had a negative PAP test. All patients with HSIL were positive for p16/Ki67, indicating a strong correlation between this biomarker and high-grade lesions. In contrast, among LSIL cases, only 15.4% were p16/Ki67 positive, and this association was not statistically significant (Fisher's exact test, $p = 1.0$).
Due to this discrepancy between LSIL findings and p16/Ki67 immunocytochemical staining, there is a risk of missing CIN2+ lesions in these patients. This highlights the need for introducing an additional method to verify precancerous lesions and cervical cancer. These findings support the diagnostic value of p16/Ki67 dual staining in identifying high-grade cervical lesions. Its use could help triage women more effectively, reducing overtreatment in low-risk cases while ensuring timely intervention for those at greater risk.

Conclusion
This study demonstrates a strong association between p16/Ki67 dual immunocytochemical staining and high-grade cervical lesions- HSIL. These findings support the use of p16/Ki67 as an adjunctive tool to improve the accuracy of cervical cancer screening and aid in the early identification of high-risk cases. Further research should aim to validate these results through histological correlation to strengthen its clinical utility.

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