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Review

Enhancing Cervical Cancer Screening: Review of p16/Ki-67 Dual Staining as a Promising Triage Strategy

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Abstract: Cervical cancer, primarily caused by high-risk human papillomavirus (HR-HPV) types 16 and 18, is a major global health concern. Persistent HR-HPV infection can progress from reversible precancerous lesions to invasive cervical cancer, which is driven by the oncogenic activity of human papillomavirus (HPV) genes, particularly E6 and E7. Traditional screening methods, including cytology and HPV testing, have limited sensitivity and specificity. This review explores the application of p16/Ki-67 dual-staining cytology for cervical cancer screening. This advanced immunocytochemical method allows for simultaneously detecting p16 and Ki-67 proteins within cervical epithelial cells, offering a more specific approach for triaging HPV-positive women. Dual staining and traditional methods are compared, demonstrating their high sensitivity and negative predictive value but low specificity. The increased sensitivity of dual staining results in higher detection rates of CIN2+ lesions, which is crucial for preventing cervical cancer progression. However, its low specificity may lead to increased false-positive results and unnecessary biopsies. The implications of integrating dual staining into contemporary screening strategies, particularly considering the evolving landscape of HPV vaccination and changes in HPV genotype prevalence are also discussed. New guidelines and further research are necessary to elucidate the long-term effects of integrating dual staining into screening protocols.

Keywords: cervical cancer; high-risk human papillomavirus; cervical intraepithelial neoplasia; Papanicolaou test; p16/Ki-67 dual staining; cytology; HPV testing

1. Introduction

Cervical cancer ranks as the fourth most prevalent malignancy among women globally and is primarily attributed to infection with high-risk human papillomavirus (HR-HPV), notably types 16 and 18 [1–8]. Persistent HR-HPV infection can progress from reversible precancerous lesions to invasive cervical cancer, driven by the actions of HPV genes, particularly E6 and E7, which lead to cell cycle dysregulation. Early detection through screening is pivotal for the prevention and management of cervical cancer [9]. Currently, cytology, HPV testing, and their combination are used for cervical cancer screening [10]. The Papanicolaou (Pap) test based on cytology has been instrumental in reducing the morbidity and mortality associated with cervical cancer [10]. However, cytology has limitations in terms of sensitivity and subjectivity, prompting the integration of HPV testing to enhance screening effectiveness [11,12].

Patients with mild cervical lesions, such as low-grade squamous intraepithelial lesions (LSIL) and atypical squamous cells of undetermined significance (ASCUS), face the risk of progression to more severe conditions, necessitating careful triage strategies [13]. Although HPV testing has high sensitivity, its low specificity leads to a significant number of unnecessary colposcopies, particularly in younger women [14]. In this context, p16/Ki-67 dual staining cytology emerges as a potential biomarker with high sensitivity and specificity for identifying high-grade cervical intraepithelial neoplasia (HGCIN) [15,16].

Although HPV testing is sensitive, it faces challenges in terms of specificity, particularly in younger populations, making it less cost-effective as a separate screening tool for women aged <30 years [17,18]. Current screening strategies involve co-testing with HPV genotyping and cytology, leading to referral for colposcopy based on abnormal findings [19]. However, effective triage tests are needed to reduce unnecessary colposcopy and enhance the detection of CIN in HPV-positive women [20]. p16/Ki-67 dual stained cytology has emerged as a promising biomarker, offering morphology-independent insights into cell cycle deregulation associated with HPV infections [21].

Although HPV testing has been a valuable addition to cervical cancer screening programs, enhancing early detection of precancerous lesions, its limitations underscore the need for additional triage strategies to optimize patient care and resource utilization [22]. This need highlights the evolving role of p16/Ki-67 dual staining cytology, which promises to address some of these limitations by offering a more specific method for triaging HPV-positive women (Table 1).

Table 1. Overview of p116/Ki-67 dual staining in cervical cancer.

Aspect	Details
Technique	Advanced immunocytochemical method for simultaneous detection of p16 and Ki-67 proteins within cervical epithelial cells.
Sensitivity	Higher sensitivity than cytology, particularly effective in detecting CIN2+ or CIN3+ lesions.
Specificity	Lower than cytology, potentially leading to more false-positive results.
Use in triage	Effective in identifying women at risk of developing high-grade cervical lesions, especially in HPV-positive cases.
Clinical implication	Facilitates early detection and management of cervical cancer, but requires cautious interpretation because of lower specificity.

2. P16/Ki-67 dual staining: an emerging tool for triage

The p16/Ki-67 dual staining technique is an advanced immunocytochemical method employed for cervical cancer screening [23]. This technique allows the simultaneous detection of p16 and Ki-67 proteins within the same cervical epithelial cell [24].

The utilization of p16INK4a in cervical cancer screening presents a promising alternative to existing triage strategies for women with abnormal Papanicolaou (Pap) results [25,26]. Current approaches, including repeat cytology, HPV testing, and colposcopy-guided biopsy, face challenges such as high referral rates and limited specificity [27]. P16INK4a, induced by HR-HPV oncogenes, exhibits a more specific association with HGCIN [28,29]. A qualitative analysis of cytological specimens, focusing on nuclear alterations, enhances specificity and enables the detection of the underlying HGCIN [30]. Studies have demonstrated that p16INK4acytology outperforms HPV testing in triaging patients with ASCUS and LSIL, demonstrating increased specificity (from approximately 50% to 80%) [26]. The p16INK4a-based approach exhibits high sensitivity and specificity for identifying HGCIN, suggesting its potential as a reliable and reproducible triage [31,32]. Its use could reduce the number of colposcopy referrals and enhance automated preselection of suspicious slides, offering an efficient and accurate tool in cervical cancer screening [33–35].

p16 is a dependent kinase inhibitor primarily involved in controlling the transition from the G1 phase to the S phase of the cell cycle [36]. This control is crucial for maintaining normal cell growth and division. In healthy cells, p16 regulates the cell cycle by inhibiting the activity of cyclin-

dependent kinases 4and 6 (CDK4/6) [37,38]. When active, these kinases phosphorylate retinoblastoma protein (pRb), a key regulator of the cell cycle. Phosphorylation of pRb leads to its inactivation, allowing the cell to progress from the G1 phase to S phase and subsequently divide [39,40]. By inhibiting CDK4/6, p16 ensures that a controlled transition occurs, thereby preventing unregulated cell proliferation [41].

In cases of high-risk HPV infection, particularly HPV types 16 and 18, the viral oncoprotein E7binds to pRb, leading to its inactivation [42]. This inactivation mimics the effect of phosphorylation and effectively removes the block that pRb typically places on the cell cycle [43]. The inactivation of pRb byE7 results in the loss of a critical control point in the cell cycle, leading to unregulated cell growth [44]. In response to this disruption, cells increase the production of p16 to counteract uncontrolled progression of the cell cycle [45,46]. Therefore, p16 overexpression is an indirect result of HPV oncogenic activity within the cell [47]. Because p16 overexpression is closely linked to the disruption of cell cycle control by high-risk HPV types, it serves as a surrogate marker of HPV-associated oncogenic activity [48]. In the context of cervical cancer screening, the detection of associated oncogenic activity has been reported [49], and the detection of elevated p16 levels indicates an ongoing HPV infection that has altered the normal regulatory mechanisms of the cell cycle, suggesting the presence of potentially precancerous or cancerous changes in cervical epithelial cells [50].

Ki-67 is a protein that is closely associated with cell proliferation and is widely used as a marker to determine the growth fraction of a cell population [51]. Its utility in the context of cervical cancer and its precursor lesions, such as cervical intraepithelial neoplasia (CIN), is particularly significant. Herein, we present a detailed examination of its role and importance [51]. Ki-67 is present in the nucleus of cells during all active phases of the cell cycle (G1, S, G2, and M phases), although it is absent in cells in the resting phase (G0) [52]. This makes Ki-67 a reliable marker for identifying actively dividing cells. The presence of this protein indicates that the cell is progressing through the cell cycle and is not in a quiescent state. In the cervical epithelium, Ki-67 expression is commonly restricted to the basal and parabasal layers, where cell division normally occurs [53]. However, in dysplastic cells, such as those observed in CIN, Ki-67 expression can be detected in the higher layers of the epithelium. This aberrant expression pattern indicates unregulated cell proliferation, which is a hallmark of precancerous and cancerous changes [54]. The level and pattern of Ki-67 expression are closely correlated with cervical dysplasia grade [55,56]. In low-grade lesions such as CIN1, Ki-67 expression may be slightly increased and remain largely confined to the lower epithelial layers. However, in high-grade lesions, such as CIN2 and CIN3, Ki-67 expression is typically more extensive and is often observed in the higher and more superficial layers of the cervical epithelium. This increase in Ki-67 staining reflects the loss of normal cell cycle control and is a key feature of CIN progression. Assessment of Ki-67 expression is valuable for both the diagnosis and prognosis of CIN and cervical cancer. Elevated Ki-67 levels in cervical cells suggest a higher rate of cell turnover, which is a characteristic of both dysplastic and neoplastic processes. Therefore, Ki-67 staining is often used in conjunction with other histopathological and cytological evaluations to assess the severity of cervical lesions [57,58]. In cervical cancer screening and management, Ki-67 can serve as an adjunct marker to improve the accuracy of cytological diagnosis. It helps differentiate between benign reactive changes and true dysplastic changes, particularly in cases where diagnosis based on morphology alone is challenging [59,60].

Dual staining for p16 and Ki-67 is particularly useful in cervical cancer screening because these two proteins are mutually exclusive in normal cells [56,61,62]. Therefore, their co-expression serves as a specific marker for HPV-mediated oncogenic transformation and indicates a higher risk of cervical cancer [58,63]. This method helps differentiate between transient, harmless HPV infections and persistent infections with a higher potential to progress to high-grade pre-cancer or invasive carcinoma.

2.2. Study outcomes on dual staining efficacy

Recent advancements in cervical cancer screening have increased the efficacy of the p16/Ki-67 dual staining technique, particularly for triaging human papillomavirus (HPV)-positive women [64,65]. A growing body of research indicates that this dual staining method outperforms traditional cytology in several key aspects, notably sensitivity and negative predictive value (NPV), albeit with a trade-off in specificity [14,56]. These findings have significant implications for early detection and management of high-grade cervical lesions.

Dual staining has been demonstrated to have significantly higher sensitivity than cytology for detecting CIN2+ or CIN3+ lesions [23,66]. This higher sensitivity indicates that dual staining is more likely to identify women with high-grade cervical lesions that could progress to cervical cancer if left undetected or untreated.

The most striking advantage of p16/Ki-67 dual staining is its heightened sensitivity for detecting CIN2+ or CIN3+ lesions, which are precursors of cervical cancer [61,64,67]. Studies have consistently indicated that the sensitivity of dual staining is markedly higher than that of traditional cytology. This increased sensitivity ensures a greater likelihood of identifying women with high-grade cervical lesions, which is critical for prompt treatment to prevent the progression to cervical cancer. The ability of dual staining to detect these lesions at an early stage can be attributed to its molecular approach that targets specific cellular changes induced by HPV infection that are occasionally missed during cytological examinations [23,55].

Another significant benefit of the dual staining technique is its higher NPV for detecting CIN2+ and CIN3+ lesions compared to cytology [56]. This specifies that a negative result from dual staining indicates a low risk of high-grade cervical lesions. In clinical practice, this translates to a reduced likelihood of overlooking significant lesions, thus providing reassurance to both patients and clinicians regarding the absence of serious pathologies [68].

However, the increased sensitivity of p16/Ki-67 dual staining was lower than that of cytology [55,64,69]. This reduced specificity may lead to more false-positive results, where the test indicates the absence of a potential lesion. Consequently, this can result in more women being referred for additional diagnostic procedures such as colposcopy and biopsy, which may not be necessary [70]. Such over-referral can cause undue anxiety in patients and may strain healthcare resources.

Despite concerns regarding specificity, the use of dual staining has been observed to increase the detection of CIN2+ cases [71]. This suggests that dual staining has a superior ability to identify significant cervical lesions compared with cytology, thereby potentially lowering the incidence of cervical cancer through early intervention. The earlier these lesions are detected and appropriately managed, the better the patient outcomes. The p16/Ki-67 dual staining technique represents a significant step forward in cervical cancer screening, particularly in HPV-positive women [72]. Its superior sensitivity and NPV for detecting high-grade lesions offers the promise of earlier and more accurate identification of women at risk of developing cervical cancer. However, the low specificity of this method necessitates cautious interpretation of the results and underscores the need for balanced clinical decision-making. As the screening landscape continues to evolve, the integration of p16/Ki-67 dual staining into existing protocols is expected to refine our approach for the detection and management of precancerous cervical conditions [73].

3. Comparative analysis of dual staining versus traditional methods

The comparative performance of p16/Ki-67 dual staining and traditional cytology methods in cervical cancer screening has significant differences in sensitivity, specificity, and predictive value (Table 2).

Table 2. Comparative analysis of screening methods [16,21,24,74–83].

Method	Sensitivity	Specificity	Suitability for triage
p16/Ki-67 dual staining	Higher	Lower	Highly suitable for HPV-positive women

| HPV resting | High | Lower, especially in younger populations | Effective, but less specific in younger women |
| Cytology | Variable, generally lower | Higher | Suitable with limitations in sensitivity |

Studies have consistently demonstrated that p16/Ki-67 dual staining exhibits higher sensitivity than traditional cytology for detecting CIN2 + and CIN3 + lesions. For instance, research indicates that the sensitivity of dual staining for detecting CIN2+ can be high at 81.8%, compared to the lower sensitivity rates for cytology. This enhanced sensitivity is pivotal for identifying additional cases of significant cervical lesions and potentially reducing the progression of cervical cancer. Although dual staining surpasses cytology in terms of sensitivity, it generally exhibits lower specificity. Dual staining may yield more false-positive results, potentially leading to more women undergoing further diagnostic procedures, such as colposcopy and biopsy, even without high-grade lesions.

The NPV of dual staining was significantly higher than that of traditional cytology, suggesting that a negative result from dual staining is more reliable in assuring a low risk of high-grade cervical lesions [84]. However, its positive predictive value may be lower than that of cytology, indicating a higher likelihood of false-positives.

The incorporation of less specific screening methods, such as HPV testing, has raised concerns regarding potential overdiagnosis and overtreatment in cervical cancer screening programs [85]. Despite the high sensitivity of high-risk HPV testing, it has lower specificity than cytology. This disparity can lead to overdiagnosis, in which HPV-positive women without significant cervical lesions are subjected to additional, possibly unnecessary, diagnostic procedures. Dual-staining has emerged as a potential solution to this challenge. Although it has a lower specificity than cytology, its high sensitivity and higher NPV make it a valuable tool for accurately identifying women at a true risk of developing high-grade lesions [86].

This could reduce the number of unnecessary colposcopies and biopsies arising from HPV testing alone [87]. The ideal screening method should strike a balance between high sensitivity (to detect as many true cases as possible) and high specificity (to minimize false positives and unnecessary interventions). Dual staining, with its high sensitivity, is beneficial for initial screening. However, its low specificity necessitates careful interpretation and additional confirmatory testing to avoid overtreatment.

The choice between dual-staining and traditional methods depends on the specific context, including patient age, HPV vaccination status, and other risk factors. Clinicians must weigh the benefits of increased sensitivity against the risks associated with lower specificity and tailor their approach to individual patient circumstances.

4. Clinical implications and future prospects

4.1. Triage strategies and follow-up results

The integration of p16/Ki-67 dual staining into cervical cancer screening protocols necessitates the re-evaluation of triage strategies, particularly in terms of follow-up and clinical management outcomes [61].

Dual staining has been demonstrated to be effective in refining the triage of HPV-positive women. With its higher sensitivity for detecting CIN2+ lesions, dual staining can identify cases that may be missed by cytology alone. However, this increased sensitivity results in a trade-off in specificity, potentially leading to more biopsies. Comparative studies indicate that the use of dual staining might result in more biopsies being performed because more women are classified as being at risk of developing CIN2+ lesions [56,61,64]. Although this increases the detection rate of significant

cervical lesions, it also increases the potential for overtreatment in cases where the lesions might regress spontaneously.

The superior sensitivity of dual staining leads to higher detection rates of CIN2+ lesions, which is crucial for preventing the progression of cervical cancer [88]. The NPV of dual staining is particularly noteworthy, as it ensures a low risk of high-grade lesions in negative cases, thus reducing unnecessary follow-up interventions.

4.2. Dual staining in contemporary screening programs

The evolving landscape of cervical cancer screening is influenced by factors such as HPV vaccination and changes in HPV genotype prevalence, and position dual staining is a potentially valuable tool in modern screening strategies [89].

As HPV vaccination programs target specific high-risk HPV types, primarily HPV 16 and 18, changes in the prevalence of other HPV genotypes are expected [90]. The role of dual-staining in identifying lesions associated with other high-risk HPV types has become increasingly important. The high sensitivity and NPV of dual staining can help streamline the process of referral to colposcopy clinics. By accurately identifying women at an actual risk of high-grade lesions, dual staining has the potential to reduce the burden on colposcopy clinics by minimizing unnecessary referrals [91]. The incidence of lesions caused by vaccine-targeted HPV will decrease in populations with a high HPV vaccination coverage. Dual staining could play a pivotal role in monitoring and managing lesions caused by non-vaccine HPV types, thereby ensuring the continued effectiveness of screening programs in the post-vaccination era. Ongoing research is essential to elucidate the long-term implications of incorporating dual staining into screening programs [23,71,80,92]. This includes evaluating cost-effectiveness, patient outcomes, and impact on healthcare systems. Additionally, guidelines for the implementation of dual staining need to be developed, considering varying healthcare contexts and population needs.

5. FAM19A4/miR1234-2 methylation testing

The hallmark of cervical carcinogenesis is hypermethylation of tumor suppressor genes. Recent clinical investigations have demonstrated that the FAM19A4/miR124-2 methylation test, labeled as CE-In Vitro Diagnostic and standardized, effectively identifies almost all cervical cancers (>98%) and reliably detects advanced CIN lesions [28,93,94]. Advanced CIN lesions, defined as CIN2/3 lesions exhibiting a cancer-like methylation profile and are linked to prolonged HPV infection may carry a heightened risk of short-term cancer progression. In women aged ≥30 years, this test exhibits a sensitivity of 77% for detecting CIN3. In a prospective clinical cohort study, the absence of FAM19A4/miR124-2 methylation is correlated with a high rate of regression in CIN2/3 lesions [95–97]. As a triage strategy, HPV16/18 genotyping is currently recommended, acknowledging the variation in carcinogenic potential among different HPV types. In young women, the FAM19A4/miR124-2 methylation test exhibited a significantly lower positivity rate for CIN2/3 than HPV16/18 genotyping, suggesting its potential to offer higher specificity in reassuring against advanced CIN lesions requiring treatment [96,97]. The ability of the test to reflect the nature of the underlying CIN, specifically distinguishing lesions with high or low short-term cancer progression risk, provides critical guidance for clinical management. With the high specificity observed in methylation-negative CIN2/3 lesions, particularly in young HPV-positive women, the test holds promise for implementing a wait-and-see policy, avoiding unnecessary overtreatment and aligning with the observed high regression rates in this population. Therefore, the FAM19A4/miR124-2 methylation test is a powerful and independent biomarker that provides detailed information for efficient and personalized therapy of cervical lesions, particularly in the context of shifting HPV vaccination guidelines [97].

The VALID-SCREEN study [95] was a retrospective EU multicenter investigation that assessed the clinical utility of the FAM19A4/miR124-2 methylation-based molecular triage test for HPV cervical cancer screening [98]. A previous study conducted on 2,384 HPV-positive cervical screening samples from women aged 29–76 years in four EU countries aimed to determine the performance of

the test as a substitute or addition to cytology in reflex testing for HPV-positive women. The FAM19A4/miR124-2 methylation test demonstrated a sensitivity of 95% for detecting screening-detected cervical cancers and an overall specificity of 78.3%. The NPV for methylation-negative outcomes in HR-HPV-positive samples was high at 99.9% for cervical cancer, 96.9% for ≥CIN3, and 93.0% for ≥CIN2. With consistent sensitivity for CIN3 across different centers, sample collection media, DNA extraction methods, and HPV screening tests, the FAM19A4/miR124-2 methylation test is an objective and potentially equivalent alternative or supplement to cytology for triaging HPV-positive women in real-life pilot implementation studies.

6. Conclusions

P16/Ki-67 dual staining stands out for its enhanced sensitivity in detecting precancerous lesions, particularly in HPV-positive women who are at a heightened risk of developing cervical cancer. The unique ability of this method to simultaneously detect p16 and Ki-67 proteins provides a more detailed and accurate assessment of the cellular changes associated with HPV-induced oncogenic activity. This feature is pivotal as it allows for the identification of lesions that are more likely to progress to higher-grade malignancies, enabling timely and targeted intervention.

Importantly, the use of p16/Ki-67 dual staining has implications beyond mere detection, offering the potential to alleviate the burden on the healthcare system. By enabling a more precise triage of patients, particularly those with HPV-positive results, unnecessary follow-ups and invasive procedures such as colposcopy and biopsy can be reduced. This efficiency not only optimizes resource utilization, but also spares patients from the anxiety and discomfort associated with these procedures.

However, the integration of p16/Ki-67 dual staining into routine clinical practice is challenging. Although sensitive, this technique exhibits lower specificity than traditional cytology, which raises the possibility of false-positive results. Therefore, its clinical application requires a balanced approach that harmonizes high sensitivity with specificity. This balance is crucial to ensure effective and economical patient-centered care, avoiding overdiagnosis and overtreatment.

As the field of cervical cancer screening continues to evolve, driven by advancements in medical technology and public health initiatives such as HPV vaccination, the role of p16/Ki-67 dual staining is expected to become more defined and integral. Future research and clinical practice should focus on refining the application of this technique and tailoring it to meet the diverse needs of patient populations and healthcare settings. This will likely involve the development of new protocols and guidelines that leverage the strengths of dual staining while addressing its limitations. In this dynamic landscape, p16/Ki-67 dual staining has emerged not only as a promising tool but also as a beacon guiding the way toward more effective, efficient, and patient-centric cervical cancer screening strategies.

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