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Diagnostic utility of p16 and Ki-67 immunohistochemical markers in cervical intraepithelial neoplasia: Association with HPV status and histological grade

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Abstract:

Background: Cervical intraepithelial neoplasia (CIN) is a precursor to cervical cancer, often associated with high-risk human papillomavirus (HPV) infection. Accurate diagnosis and grading of CIN are critical for effective management. Immunohistochemical markers like p16 and Ki-67 can enhance diagnostic precision by identifying oncogenic HPV-induced changes and cellular proliferation. This study evaluates the diagnostic utility of p16 and Ki-67 expression in CIN lesions and association with HPV status and histological grade.
Methodology: This retrospective study analyzed archived cervical biopsy samples diagnosed with CIN over a 5-year period in a tertiary care center in Sagamu, Ogun State, Nigeria. Histological grading of CIN was confirmed, and immunohistochemical staining for p16 and Ki-67 was performed. HPV status was determined using polymerase chain reaction (PCR) on the biopsy sample. Marker expression patterns were compared across CIN grades and with HPV status. Statistical association of p16 and Ki-67 expression with CIN grades and HPV was done using Chi-square or Fisher Exact test, with $p<0.05$ considered as statistical significance.
Results: Of the 72 samples analyzed, p16 expression showed a significant association with higher CIN grades and HPV positivity ($p<0.001$), with sensitivity and specificity values of 92.0% and 88.0%, respectively. Ki-67 expression was also associated with increasing CIN grades and HPV status ($p=0.001$), and demonstrated a sensitivity of 85.0% and specificity of 82.0% in distinguishing CIN lesions.
Conclusion: p16 and Ki-67 immunohistochemical markers are valuable adjuncts in the diagnosis and grading of CIN, particularly when correlated with HPV status. Their combined use can enhance diagnostic accuracy, supporting their incorporation into routine pathology workflows for CIN evaluation.

Keywords: Biomarkers; cervical biopsy; CIN; HPV; Ki67; p16

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Utilité diagnostique des marqueurs immunohistochimiques p16 et Ki-67 dans les néoplasies intraépithéliales cervicales: Association avec le statut HPV et le grade histologique

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Résumé:

Contexte: La néoplasie intraépithéliale cervicale (CIN) est un précurseur du cancer du col de l'utérus, souvent associée à une infection par le virus du papillome humain (VPH) à haut risque. Un diagnostic et une classification précis de la CIN sont essentiels à une prise en charge efficace. Les marqueurs immuno-histochimiques tels que p16 et Ki-67 peuvent améliorer la précision du diagnostic en identifiant les modifications oncogènes induites par le VPH et la prolifération cellulaire. Cette étude évalue l'utilité diagnostique de l'expression de p16 et Ki-67 dans les lésions de CIN et leur association avec le statut VPH et le

grade histologique.
Méthodologie: Cette étude rétrospective a analysé des échantillons de biopsies cervicales archivés chez lesquels une CIN a été diagnostiquée sur une période de 5 ans dans un centre de soins tertiaires de Sagamu, dans l'État d'Ogun, au Nigéria. La classification histologique de la CIN a été confirmée et une coloration immunohistochimique pour p16 et Ki-67 a été réalisée. Le statut HPV a été déterminé par PCR (amplification en chaîne par polymérase) sur l'échantillon de biopsie. Les profils d'expression des marqueurs ont été comparés entre les grades de CIN et le statut HPV. L'association statistique de l'expression de p16 et Ki-67 avec les grades de CIN et le HPV a été réalisée par test du Chi carré ou test exact de Fisher, avec une valeur de $p<0,05$ considérée comme statistiquement significative.
Résultats: Sur les 72 échantillons analysés, l'expression de p16 a montré une association significative avec des grades de CIN plus élevés et une positivité HPV ($p<0,001$), avec des valeurs de sensibilité et de spécificité de 92,0% et 88,0%, respectivement. L'expression de Ki-67 a également été associée à des grades de CIN plus élevés et au statut HPV ($p=0,001$), et a démontré une sensibilité de 85,0% et une spécificité de 82,0% pour distinguer les lésions de CIN.
Conclusion: les marqueurs immunohistochimiques p16 et Ki-67 sont des compléments précieux pour le diagnostic et la classification des CIN, en particulier lorsqu'ils sont corrélés au statut HPV. Leur utilisation combinée peut améliorer la précision du diagnostic, favorisant ainsi leur intégration dans les procédures de routine de pathologie pour l'évaluation des CIN.

Mots-clés: Biomarqueurs; biopsie cervicale; CIN; HPV; Ki67; p16

Introduction:

Cervical cancer remains a significant public health challenge (1), particularly in low-and-middle-income countries where it is a leading cause of cancer-related morbidity and mortality among women (2). Persistent infection with high-risk human papillomavirus (HPV) is recognized as the primary etiological factor in the development of cervical intraepithelial neoplasia (CIN) and its progression to invasive carcinoma (3). Early detection and appropriate management of CIN are pivotal in preventing cervical cancer, underscoring the importance of accurate diagnostic techniques (4).

Histopathological examination of cervical biopsies remains the gold standard for diagnosing CIN (4), however, the subjective interpretation of histology can lead to observers' variability, particularly in distinguishing between low-grade (CIN I) and high-grade (CIN II/III) lesions (5). The advent of immunohistochemical markers, such as p16 and Ki-67, offers a promising adjunct to traditional histopathology by providing objective and reproducible insights into the biological behavior of these lesions (6).

As a surrogate marker for oncogenic HPV activity, p16 reflects the overexpression of the CDKN2A gene, which occurs due to HPV-mediated inactivation of tumor suppressor proteins. Ki-67, a nuclear protein expressed during active phases of the cell cycle, indicates cellular proliferation and abnormal growth patterns (6). Individually, these markers have demonstrated utility in distinguishing CIN from benign and reactive lesions. Combined, they provide complementary information, enhancing diagnostic precision (7). Despite the availability of these markers, their integration into routine practice varies, particularly in resource-limited settings (6). Furthermore, the relationship between p16 and Ki-67 expression, HPV status, and CIN grade requires further exploration to optimize diagnostic algorithms (7).

In recent years, the combination of p16 and Ki-67 has gained attention as a reliable method for improving the accuracy of CIN diagnosis and grading (8). However, the diagnostic performance of these markers in different clinical settings and populations remains under-explored (9). This study aims to address this gap by evaluating the role of p16 and Ki-67 in diagnosing CIN and exploring their correlation with HPV status and histological grading. By providing a clearer understanding of these markers' diagnostic utility, this research seeks to contribute to the refinement of screening and diagnostic protocols, particularly in settings with limited access to advanced molecular testing.

Materials and method:

Study setting and design:

A retrospective study was conducted in the Department of Morbid Anatomy and Histopathology at Olabisi Onabanjo University Teaching Hospital, Sagamu, southwest Nigeria, covering the period from December 2019 to November 2024. The study was approved by the ethical review board of the hospital, and patient confidentiality was maintained throughout the study.

Study population:

The study included archived formalin-fixed, paraffin-embedded (FFPE) cervical biopsy specimens from women diagnosed with cervical intraepithelial neoplasia (CIN) during the study period. Cases without adequate histological samples or complete clinical data were excluded from the study to ensure accuracy.

Histopathological grading:

Cervical biopsy specimens were examined and reviewed microscopically. They were graded as CIN lesions according to the

Bethesda system (10). CIN lesions were classified as CIN I (mild dysplasia), CIN II (moderate dysplasia), or CIN III (severe dysplasia/carcinoma in situ).

Immunohistochemical staining:

Immunohistochemical staining for the p16 and Ki-67 was performed on all eligible specimens. Briefly, 4-µm thick sections were cut from FFPE blocks and mounted on slides. For p16, the slides were incubated with a monoclonal antibody (clone E6H4, Dako, Denmark) at a dilution of 1:100. For Ki-67, the slides were incubated with a monoclonal antibody (clone MIB-1, Dako, Denmark) at a dilution of 1:200. Following incubation, the slides were processed with appropriate secondary antibodies, and the results were visualized using 3,3'-diaminobenzidine (DAB) as the chromogen. The slides were counterstained with hematoxylin.

Scoring and interpretation of immunohistochemical staining:

The expression of p16 and Ki-67 was assessed by the percentage of positive cells and the intensity of staining. p16 expression was considered positive when more than 70% of the cells showed strong diffuse staining, consistent with the overexpression typical of HPV-induced lesions. Ki-67 expression was scored based on the percentage of positive cells in the epithelial layer, with a cutoff of >10% staining indicating high proliferative activity. Both markers were classified as positive or negative based on these criteria.

HPV testing:

HPV testing was performed on cervical biopsy specimens using polymerase chain reaction (PCR) to detect the presence of high-risk HPV types (16, 18, and other high-risk strains). HPV DNA was extracted from FFPE tissue sections using the Qiagen DNA extraction kit (Qiagen, Germany) following the manufacturer's instructions.

Amplification by PCR was performed using type-specific primers targeting the L1 region of high-risk HPV genotypes, including HPV types 16, 18, 31, 33, and 45. Each 25 µL PCR reaction mixture contained 10–100 ng of extracted DNA, 0.2 µM of each primer, 200 µM dNTPs, 1.5 mM $MgCl_2$, 1× PCR buffer, and 1 unit of Taq DNA polymerase. Thermal cycling conditions included an initial denaturation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 45 seconds, and a final extension at 72°C for 7 minutes.

PCR products were analyzed by electrophoresis on a 2% agarose gel stained with ethidium bromide and visualized under UV illumination. A sample was considered HPV-positive if at least one high-risk HPV genotype was detected.

Data analysis:

Descriptive statistics were used to summarize patient demographics and clinicopathological features. The association between p16 and Ki-67 expression and CIN grade was analyzed using the Chi-square test or Fisher's exact test, as appropriate. The sensitivity and specificity for each marker and their combined use in diagnosing CIN were calculated. A *p*-value of <0.05 was considered statistically significant.

Results:

Clinicopathological characteristics of the patients with cervical biopsy samples:

As depicted in Table 1, archived cervical biopsy samples of a total of 72 patients were involved in the study. The mean age (standard deviation) of the patients is 31.61 ±8.75 years, with predominant age group between 21-40 years (n=58 cases, 80.6%).

All samples were positive for HPV except 5 (6.9%), with low-risk (LR)-HPV (n= 37, 51.4 %) and high-risk (HR)-HPV cases (n=30, 41.7 %). CIN I was the diagnosis in 37 cases (51.4 %), while CIN II and CIN II were seen in 19 cases (26.4 %) and 16 cases (22.2 %) respectively.

Table 1: Clinicopathological characteristics of women whose cervical samples were used in the study

Parameter	Frequency (%)
Age group (years)	
1-20	5 (6.9)
21-40	58 (80.6)
41-60	9 (12.5)
Mean age (± SD)	31.60 ± 8.75
HPV status	
HPV negative	5 (6.9)
LR HPV	37 (51.4)
HR HPV	30 (41.7)
CIN grade	
CIN I	37 (51.4)
CIN II	19 (26.4)
CIN III	16 (22.2)

CIN=cervical intraepithelial neoplasia; HPV=Human papilloma virus, LR=Low-risk; HR-High-risk; SD=Standard deviation

Association between p16 expression and CIN grades:

A significant association was found between expression of p16 with increased severity of the cervical lesion ($p<0.001$) as showed in Table 2. Only cases of CIN I were negative for p16 (5.4%, 2/37), with 27 of the 37 (73.0%) CIN I cases showing weak staining (+). In contrast, the majority of CIN II cases showed intermediate staining (++) intensity (9/19, 47.4%) while most of CIN III cases showed strong staining (+++) intensity

for p16 (13/16, 81.3%). There was no strong staining for p16 amongst the CIN I cases.

Association between Ki67 expression and CIN grades:

As illustrated in Table 3, a significant association was found between Ki-67 expression and increase in the severity of the cervical lesion (p=0.001). Most cases of CIN I showed weak p16 stain (24/37, 64.9%). In contrast, most of CIN II showed intermediate staining (9/19, 47.4%) and strong staining (8/19, 42.1%), while the majority of CIN III cases showed strong staining (11/16, 68.8%). CIN III did not show weak staining at all, while only 2 cases (2/19, 10.5%) of CIN II showed weak staining.

Association between p16 and Ki67 staining patterns:

Cervical samples with intermediate or strong intensity for p16 did not show negativity or weak staining for Ki-67 as illustrated in Table 4. Cases negative for p16 did not show intermediate or strong staining for Ki-67, however, 1 of the 2 (50.0%) p16 negative staining showed a weak staining pattern for Ki-67. Cases with weak staining for p16 did not show strong staining for Ki-67, however, they showed mostly weak staining (23/30, 76.7%) and to a lesser extent, intermediate staining (7/30, 23.3%), and this finding was significantly associated (p<0.001)

Association between HPV status and CIN grades:

As shown in Table 5, 5 patients with a history of CIN I were negative for HPV while majority were positive for LR-HPV (30/37, 81.1%). Patients with history of CIN II and CIN III mostly showed positivity for HR-HPV (13/19, 68.4%) and (15/16, 93.8%), and this was significantly associated (p=0.001).

Association between HPV status and p16 and Ki67 staining score:

As observed in Table 6, a significant association was found between HPV genotype and the expression of either p16 or Ki-67 (p<0.001 for each). HPV negative cases did not show intermediate or strong staining scores for either p16 or Ki67 but showed weak staining for p16 (3/5, 60.0%) and Ki-67 (4/5, 80%). Most of HR-HPV positive cases showed strong staining score (+++) for both p16 (19/30, 63.3%) and Ki67 (17/30, 56.7%) with no negative staining score for both. LR-HPV positive cases mostly showed weak staining pattern for p16 (23/37, 62.2%) or Ki-67 (18/37, 48.6%) or showed intermediate staining pattern for p16 (13/37, 35.1%) and Ki-67 (17/37, 45.9%).

Table 2: Association of p16 expression with cervical intraepithelial neoplasia grade

CIN grades	**No of sample with p16 expression level (%)**					***p* value**
	-	**+**	**++**	**+++**	**Total**	
CIN I	2 (5.4)	27 (73.0)	8 (21.6)	0	**37 (51.4)**	<0.001*
CIN II	0	3 (15.8)	9 (47.4)	7 (36.8)	**19 (26.4)**	
CIN III	0	0	3 (18.8)	13 (81.3)	**16 (22.2)**	
Total	**2 (2.8)**	**30 (41.7)**	**20 (27.8)**	**20 (27.8)**	**72 (100.0)**	

* = Statistically significant; + = weak staining; ++ = moderate staining; +++ = strong staining; - = no staining

Table 3: Association of Ki-67 expression and cervical intraepithelial neoplasia grade

CIN grades	**No of samples with Ki-67 expression level (%)**					***p* value**
	-	**+**	**++**	**+++**	**Total**	
CIN I	1 (2.7)	24 (64.9)	12 (32.4)	0	**37 (51.4)**	<0.001*
CIN II	0	2 (10.5)	9 (47.4)	8 (42.1)	**19 (26.4)**	
CIN III	0	0	5 (31.3)	11 (68.8)	**16 (22.2)**	
Total	**1 (1.4)**	**26 (36.1)**	**26 (36.1)**	**19 (26.4)**	**72 (100)**	

* = Statistically significant; + = weak staining; ++ = moderate staining; +++ = strong staining; - = No staining

Table 4: Association between p16 and Ki-67 expression levels

No of samples with p16 expression level (%)	**No of samples with Ki-67 expression level (%)**					***p* value**
	-	**+**	**++**	**+++**	**Total**	
-	1 (50.0)	1 (50.0)	0	0	**2**	<0.001*
+	0	23 (76.7)	7 (23.3)	0	**30**	
++	0	2 (10.0)	17 (85.0)	1 (5.0)	**20**	
+++	0	0	2 (10.0)	18 (90.0)	**20**	
Total	**1**	**26**	**26**	**19**	**72**	

* = Statistically significant; + = weak staining; ++ = moderate staining; +++ = strong staining; - = No staining

Table 5: Association of human papillomavirus status with cervical intraepithelial neoplasia grade

CIN grades	**HPV status (%)**				*p* value
	HPV –ve	**LR-HPV**	**HR-HPV**	**Total**	
CIN I	5 (13.5)	30 (81.1)	2 (5.4)	**37 (51.4)**	<0.001*
CIN II	0	6 (31.6)	13 (68.4)	**19 (26.4)**	
CIN III	0	1 (6.2)	15 (93.8)	**16 (22.2)**	
Total	**5 (6.9)**	**37 (51.4)**	**30 (41.7)**	**72 (100.0)**	

* = Statistically significant; CIN=cervical intraepithelial neoplasia; HPV=Human papilloma virus, LR=Low-risk; HR-High-risk

Table 6: Association of HPV status with p16 and Ki-67 intensity scores

HPV	**No of samples with p16 expression level (%)**				**No of samples with Ki-67 expression level (%)**			
	-	**+**	**++**	**+++**	**-**	**+**	**++**	**+++**
HPV–ve (n=5)	2 (40.0)	3 (60.0)	0	0	1 (20.0)	4 (80.0)	0	0
LR-HPV (n=37)	0	23 (62.2)	13 (35.1)	1 (2.7)	0	18 (48.6)	17 (45.9)	2 (5.4)
HR-HPV (n=30)	0	4 (13.3)	7 (23.3)	19 (63.3)	0	4 (13.3)	9 (30.0)	17 (56.7)
p value	<0.001*				<0.001*			

* = Statistically significant; + = weak staining; ++ = moderate staining; +++ = strong staining; - = No staining; CIN=cervical intraepithelial neoplasia; HPV=Human papilloma virus, LR=Low-risk; HR-High-risk

Discussion:

This study aimed to evaluate the diagnostic utility of p16 and Ki-67 immunohistochemical markers in the grading of CIN in relation to HPV genotype. The results demonstrated significant associations between p16 and Ki-67 expression and CIN grades, with strong associations between HR HPV infection and higher CIN grades. These findings are consistent with the growing body of evidence that highlights the role of immunohistochemical markers in the early diagnosis and grading of cervical lesions (11,12).

In the present study, a significant association (p<0.001) was detected between p16 expression and the severity of cervical lesion; the strongest staining (+++) was most frequently found in CIN III (65.0%), intermediate staining (++) was mostly detected in CIN II (45.0%), while no cases of CIN I showed p16 strong staining score. This result agrees with previous studies which reported that diffuse intense p16 expression was found in 88-100% of invasive carcinoma, 40- 100% of CIN II/III, and 0-15% in non-dysplasia (13,14,15). One study which reported a higher rate of p16 expression in CINs and invasive cancer also found a higher rate of expression in non-dysplasia (32%) than other studies (16), which may be attributed to the criteria used in that study or to HPV infection genotypes.

This study also reported a significant association between ki-67 expression and severity of the cervical lesion (p=0.001). CIN II and CIN III showed strong positive staining for Ki-67 (42.1% and 68.8% respectively) while cases of CIN I show 0%. These results agree with what was reported earlier that Ki-

67 expression was seen in 90-100% of invasive carcinoma, 20-70% in CIN II/III, 70-90% in CIN I, and 0-20% in non-dysplasia (13,14,15). Immunopositivity for Ki-67 linearly increases as the CIN grade is higher (17). A study by Kruse et al., (18) also found Ki-67 to be useful in distinguishing different grades of CIN lesions, and a potential method of quality control and possible indicator of progression in low-grade lesions.

A significant positive association was found between combined p16 and Ki67 expression in this study (p<0.000). The expression levels of p16 and Ki67 gradually increase with the development of CIN lesions and cervical cancer as previously reported (17). Moreover, p16/Ki67 dual staining has been used for cervical cancer screening (19). The current study revealed that most patients with CIN I are either negative for HPV or positive for LR-HPV (13.5% and 81.1% respectively), while patients with CIN II and CIN III are mostly positive for HR-HPV (68.4%, and 93.8% respectively) and this association was statistically significant (p=0.001). These results agree with what was reported by Nam et al., (20) who showed that in low-grade CIN, HPV test was negative in 54.5% of patients. HPV positivity rates tend increase directly with the cervical lesion severity (17).

Regarding the association of p16 and Ki-67 with HPV status, our study showed that HPV negative cases did not have intermediate or strong staining score for either p16 or Ki-67 while most of the HR-HPV positive cases had strong positive staining for both p16 and Ki-67and this was statistically significant (p <0.001 for each). This agrees with a study by Bosch et al., (21), who found that the p16 over-expression is rare in patients infected with a low-risk HPV because E7 protein of low-risk HPV has a lower affinity for pRb than that of high-risk HPV. Klaes et al., (22) found that over-expression of p16 has a close association with high-risk HPV infection and correlates with the degree of cervical neoplasia. Keating et al., (23) also showed a strong relationship between Ki-67, cyclin E, and p16 in the recognition of HPV-associated precursors as well as in distinguishing normal squamous mucosa from precancerous lesions and, found that with the increase of p16 and Ki-67 expression levels, the number of HR-HPV copies increase.

Several studies have highlighted the diagnostic value of p16 and Ki-67 in distinguishing high-grade CIN (CIN II/III) from low-grade lesions (CIN I). In our study, p16 demonstrated high sensitivity and specificity for high-grade lesions, consistent with its established role as a surrogate marker for transforming high-risk HPV infection. Ki-67 also showed increased expression with rising CIN grade, supporting its utility as a proliferation marker. Previous studies have reported sensitivities ranging from 86% to 100% and specificities between 70% and 95% for p16 in detecting CIN II/III lesions (8,17,20). Ki-67, while slightly less specific when used alone, provides added diagnostic value when co-expressed with p16, especially in ambiguous or borderline cases. The combined use of p16 and Ki-67 has been shown to enhance diagnostic accuracy and interobserver agreement, offering a reliable adjunct to routine histology. These findings support the incorporation of both markers into routine practice for more accurate grading of CIN.

Conclusion:

This study highlights the diagnostic value of p16 and Ki-67 immunohistochemical markers in the assessment of CIN, particularly in distinguishing high-grade lesions (CIN II and CIN III) from low-grade lesions (CIN I). The significant association between these biomarkers and CIN grade, coupled with the strong association of HR HPV with high-grade lesions, highlights the utility of a multi-marker approach in the diagnostic and prognostic evaluation of cervical dysplasia.

Our results also suggest that both p16 and Ki-67 are highly accurate in identifying high-grade CIN lesions, with p16 demonstrating slightly higher diagnostic accuracy. The combined use of p16 and Ki-67 may improve the overall diagnostic performance in distinguishing between low- and high-grade lesions. Future studies should aim to further validate these findings in larger cohorts and explore the potential role of p16 and Ki-67 in monitoring disease progression and predicting the risk of cervical cancer.

Contribution of authors:

DTO conceived and designed the study, acquired, analyzed and interpreted the data. HAS acquired and interpreted data. OS and SK interpreted the data. All the authors drafted, revised, read and approved the manuscript.

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No conflict of interest is declared

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