Section: Miscellaneous

Original Research Article

p16INK4a AS AN ADJUNCT TO HISTOPATHOLOGY IN THE DIAGNOSIS OF PRECANCEROUS AND CANCEROUS LESIONS OF THE UTERINE CERVIX

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ABSTRACT

Background: Cervical cancer continues to be a significant public health concern, particularly in developing countries, where late diagnosis and limited screening contribute to high morbidity and mortality. The identification of reliable biomarkers is essential to improve diagnostic accuracy in cervical lesions. The objective is to evaluate the role of p16INK4a as an adjunct to histopathology in the diagnosis and grading of precancerous and malignant lesions of the uterine cervix.

Materials and Methods: This observational study included 100 cervical biopsy specimens encompassing benign lesions, cervical intraepithelial neoplasia (CIN I–III), and invasive carcinoma. All cases were subjected to routine histopathological examination using hematoxylin and eosin staining, followed by immunohistochemical analysis for p16INK4a expression. Statistical analysis was performed using the chi-square test, Fisher's exact test, and logistic regression.

Results: A progressive increase in p16INK4a expression was observed with increasing lesion severity, with positivity noted in 66.6% of CIN I, 87.5% of CIN II, 92% of CIN III, and 100% of carcinoma cases, while all benign lesions were negative. A statistically significant association was found between p16INK4a expression and lesion grade ($p < 0.0001$). Staining intensity and the proportion of positive cells showed a strong correlation with disease progression. The biomarker demonstrated high diagnostic performance, with sensitivity increasing from low-grade to high-grade lesions and specificity consistently at 100%. Logistic regression analysis identified staining intensity as an independent predictor of higher-grade lesions.

Conclusion: p16INK4a is a reliable and effective adjunct biomarker that enhances diagnostic accuracy in cervical lesions. Its strong correlation with lesion severity and high specificity makes it particularly valuable in differentiating benign, premalignant, and malignant conditions, as well as in guiding prognostic assessment.

Keywords: Cervical cancer, p16INK4a, CIN, HPV, Immunohistochemistry, Biomarker.

INTRODUCTION

Cervical cancer remains a significant global public health challenge and continues to be a leading cause of cancer-related morbidity and mortality among women, particularly in developing countries. The burden is disproportionately high in low-resource settings, where inadequate access to organised screening programs, limited awareness, and delayed clinical presentation contribute to late-stage diagnosis and poor outcomes. Regional data from Eastern Uttar Pradesh further underscore persistent gaps in knowledge, suboptimal screening uptake, and the prevalence of modifiable risk factors, highlighting the urgent need for improved diagnostic

and preventive strategies (Arbyn et al., 2020; Sung et al., 2021; Yadav et al., 2023).[1-3]

Persistent infection with high-risk human papillomavirus (HPV), particularly genotypes 16 and 18, is well established as the primary etiological factor in cervical carcinogenesis. The viral oncoproteins E6 and E7 play a central role in tumor development by inactivating key tumor suppressor pathways, including p53 and retinoblastoma protein (pRb), thereby promoting uncontrolled cellular proliferation and progression from cervical intraepithelial neoplasia (CIN) to invasive carcinoma (Doorbar et al., 2012; Schiffman et al., 2016).[4,5] In addition to viral oncogenesis, host-related factors such as genetic susceptibility also contribute to disease progression. Polymorphisms, including methylenetetrahydrofolate reductase (MTHFR) C677T, have been associated with an increased risk of cervical cancer, suggesting a multifactorial pathogenesis involving both environmental and genetic determinants (Devi et al., 2022).[6]

Cytological screening methods, particularly the Papanicolaou (Pap) smear, have substantially reduced the incidence of cervical cancer in high-income countries. However, their diagnostic accuracy remains limited by sampling errors, subjective interpretation, and interobserver variability. These limitations are especially relevant in distinguishing low-grade lesions from high-grade lesions with significant malignant potential (Wentzensen et al., 2017).[7] Consequently, there is a growing need for objective, reproducible biomarkers that can complement conventional histopathological evaluation and enhance diagnostic precision.

In this context, p16INK4a has emerged as a robust biomarker for HPV-associated cervical lesions. Its overexpression results from functional inactivation of the pRb pathway by HPV E7 oncoprotein, making it a reliable surrogate marker of transforming HPV infections. Accumulating evidence demonstrates a strong correlation between p16INK4a expression and the severity of cervical epithelial abnormalities, thereby facilitating the differentiation of benign lesions from premalignant and malignant conditions (Cuschieri et al., 2014; Darragh et al., 2012).[8,9]

Given these considerations, the present study was designed to evaluate the diagnostic utility of p16INK4a as an adjunct to histopathology in cervical lesions. Specifically, it aims to assess its expression across different grades of cervical intraepithelial neoplasia and to determine its effectiveness in distinguishing benign, premalignant, and malignant cervical lesions.

MATERIALS AND METHODS

Study Design and Setting: This observational study was conducted over a period of three years in the Department of Pathology in collaboration with the Department of Obstetrics and Gynaecology at Nehru Chikitsalaya, B.R.D. Medical College, Gorakhpur. Ethical approval was obtained from the Institutional Ethics Committee, and written informed consent was obtained from all participants in accordance with the principles of the Declaration of Helsinki (World Medical Association, 2013).

Study Population and Case Selection: A total of 100 cases were included through both prospective and retrospective enrollment. The study population comprised patients presenting with a spectrum of cervical lesions, including benign conditions, cervical intraepithelial neoplasia (CIN), and invasive carcinoma.

Inclusion Criteria

- Women aged 21–70 years
- Clinically suspected precancerous or malignant cervical lesions
- Patients providing written informed consent

Exclusion Criteria

- Cases not adhering to study protocol
- Autolysed or inadequate tissue samples lacking both ectocervical and endocervical components

Histopathological Examination: Cervical biopsy and hysterectomy specimens were fixed in 10% neutral buffered formalin and processed using standard histopathological techniques, including dehydration in graded alcohol, clearing in xylene, and paraffin embedding (Bancroft & Gamble, 2019).[10] Paraffin-embedded tissues were sectioned at 4–5 µm thickness using a rotary microtome and stained with Hematoxylin and Eosin (H&E) for routine histopathological evaluation. Lesions were categorized according to the World Health Organization (WHO) classification of female genital tumors before immunohistochemical analysis (WHO Classification of Tumours Editorial Board, 2020).[11]

Immunohistochemistry for p16INK4a

Immunohistochemical staining for p16INK4a was performed on 4–5 µm thick sections mounted on poly-L-lysine-coated slides using a mouse monoclonal antibody (Clone G175-405, BioGenex).[12]

Antigen Retrieval and Pre-treatment

Sections were deparaffinized in xylene and rehydrated through graded alcohols. Antigen retrieval was carried out using heat-induced epitope retrieval in citrate buffer (pH 6.0) using a microwave method. Slides were allowed to cool and then rinsed with phosphate-buffered saline (PBS, pH 7.4) (Shi et al., 2011).[13]

Immunostaining Procedure

Immunostaining was performed using the BioGenex Super Sensitive™ Polymer-HRP detection system according to the manufacturer's instructions. Endogenous peroxidase activity was blocked, followed by application of a protein block to reduce non-specific binding. Sections were incubated with the primary antibody, followed by secondary detection reagents, including enhancer and polymer-HRP.

Visualization was achieved using 3,3′-diaminobenzidine (DAB) as chromogen, and sections were counterstained with hematoxylin, dehydrated,

cleared, and mounted (Ramos-Vara & Miller, 2014).[14]

Interpretation of Immunohistochemical Staining

p16INK4a expression was evaluated based on nuclear and cytoplasmic staining patterns, intensity, and proportion of positive cells. Interpretation was performed in accordance with established criteria for p16 immunoreactivity in cervical lesions (Darragh et al., 2012). Findings were correlated with histopathological diagnosis for diagnostic assessment. Appropriate positive and negative controls were included to validate staining results.

Statistical Analysis: Data were analysed using standard statistical software (e.g., SPSS version 25.0; IBM Corp., Armonk, NY). Descriptive statistics were used to summarise the data. The association between p16INK4a expression and histopathological grading was assessed using the Chi-square test.

Spearman's rank correlation was applied to evaluate the relationship between p16 expression and lesion severity. Logistic regression analysis was performed to identify independent predictors of high-grade lesions, including staining intensity, percentage positivity, and staining pattern. A p-value of <0.05 was considered statistically significant (Altman, 1991).[15]

RESULTS

Clinico-demographic Profile: A total of 100 cases of cervical lesions were included in the study. The age ranged from 21 to 70 years, with a mean age of 43.72 years. The majority of cases were observed in the 31–40 years age group (36%), followed by 41–50 years (32%). Most patients were from rural areas (66%). However, higher parity (≥3) showed a strong association with increasing lesion severity. The majority of CIN II (62.5%), CIN III (80%), and carcinoma (85.72%) cases were observed in multiparous women. Although benign lesions most commonly presented with vaginal discharge (64.28%). In contrast, CIN lesions predominantly presented with abnormal vaginal bleeding, while postmenopausal bleeding was the most common presenting complaint in invasive carcinoma (57.14%).

Histopathological Spectrum: [Table 1] histopathological evaluation revealed a spectrum of cervical lesions, with CIN III constituting the largest group (25%), followed by CIN I (24%), carcinoma (21%), CIN II (16%), and benign lesions (14%). Squamous cell carcinoma was the predominant subtype (90.47%), followed by adenocarcinoma (9.52%) and adenosquamous carcinoma (4.76%).

p16INK4a Expression Profile: Immunohistochemical analysis demonstrated a progressive increase in p16INK4a expression with lesion severity. All benign lesions were negative, while positivity was observed in 66.6% of CIN I, 87.5% of CIN II, 92% of CIN III, and 100% of carcinoma cases [Table 2].

Table 1: Histopathological distribution

Lesion Type	Cases (n)	Percentage (%)
Benign	14	14%
CIN I	24	24%
CIN II	16	16%
CIN III	25	25%
Carcinoma	21	21%

Table 2: p16INK4a expression across lesions

Lesion Type	Total Cases	Positive Cases	Positivity (%)
Benign	14	0	0%
CIN I	24	16	66.6%
CIN II	16	14	87.5%
CIN III	25	23	92%
Carcinoma	21	21	100%

The combined histopathological and immunohistochemical evaluation demonstrated a clear correlation between lesion grade and p16INK4a expression, with a progressive increase in staining intensity and distribution from benign to malignant lesions. Benign cervical tissues, including normal epithelium and chronic cervicitis, showed preserved stratified squamous architecture with lymphoplasmacytic infiltration in inflammatory lesions. These cases consistently exhibited absent p16INK4a expression, confirming their non-neoplastic nature [Figure 1].

CIN I lesions demonstrated mild cytonuclear atypia confined to the lower one-third of the epithelium, with focal p16INK4a positivity limited to basal and parabasal layers. CIN II lesions showed moderate dysplasia involving the lower two-thirds of the epithelium, accompanied by increased staining intensity with continuous nuclear and cytoplasmic p16INK4a expression. CIN III lesions exhibited full-thickness epithelial dysplasia with marked nuclear atypia and hyperchromasia. These lesions demonstrated strong and diffuse p16INK4a expression [Figure 2] throughout the epithelial thickness. Invasive carcinomas showed stromal invasion with marked cellular atypia. Histological subtypes included squamous cell carcinoma and adenosquamous carcinoma. All malignant cases demonstrated strong, diffuse, and continuous nuclear and cytoplasmic p16INK4a expression, irrespective of subtype.

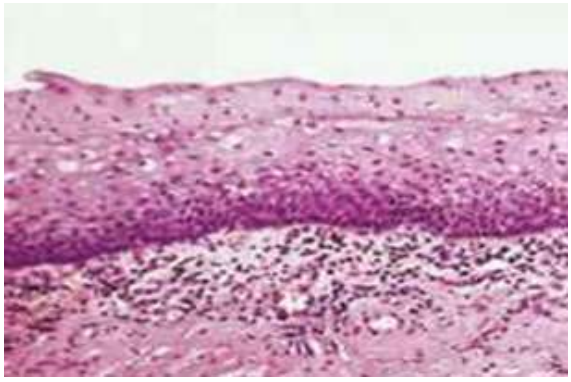

Figure 1 Chronic cervicitis, showing uniform inflammation within the cervical mucosa. (H&E stain).

Quantitative Expression (Cell Positivity)

A progressive increase in the percentage of p16INK4a-positive cells was noted with increasing lesion severity [Table 3]. All benign lesions showed grade 0 staining. CIN I demonstrated variable expression, whereas higher grades (CIN II, CIN III, and carcinoma) showed predominance of grade 2 and grade 3 positivity.

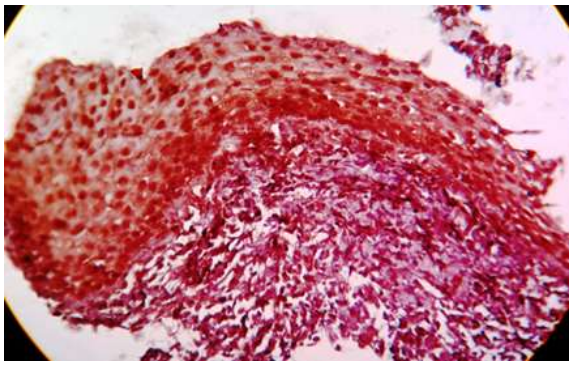

Figure 2: p16INK4a immunostaining reveals strong, diffuse, and continuous nuclear and cytoplasmic positivity, consistent with high-grade lesion/invasive carcinoma.

Table 3: Percentage positivity grading

Lesion Type	Grade 0	Grade 1	Grade 2	Grade 3
Benign	100%	0	0	0
CIN I	33.4%	16.6%	11.1%	37.5%
CIN II	12.5%	25%	37.5%	25%
CIN III	8%	0	44%	48%
Carcinoma	0	0	14.28%	85.72%

Staining Intensity & Pattern: Staining intensity showed a progressive increase with lesion severity. All benign lesions demonstrated negative staining, while CIN lesions showed weak to moderate intensity. Strong staining was predominantly observed in CIN III and carcinoma cases [Table 4]. Diffuse nuclear and cytoplasmic staining patterns were more frequent in high-grade lesions and carcinoma, whereas focal staining was characteristic of low-grade lesions.

Table 4: Staining intensity distribution

Lesion Type	Negative	Weak	Moderate	Strong
Benign	100%	0	0	0
CIN I	33.3%	25%	20.8%	20.8%
CIN II	12.5%	37.5%	37.5%	12.5%
CIN III	8%	0	64%	28%
Carcinoma	0	0	47.6%	52.4%

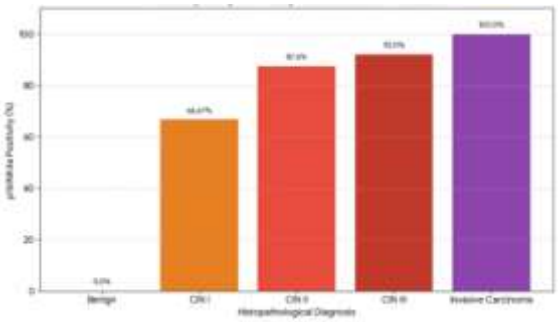

Figure 3: Shows a clear linear increase in p16INK4a expression across cervical lesions, rising from 0% in benign cases to 66.67% in CIN I, 87.5% in CIN II, 92% in CIN III, and 100% in invasive carcinoma. This progressive trend highlights a strong association between p16INK4a positivity and lesion severity, supporting its role as a marker of disease progression.

Linear Analysis: A statistically significant linear increase in p16INK4a expression was observed across the ordered spectrum of cervical lesions, ranging from benign lesions to invasive carcinoma. Expression was absent in benign lesions (0%), became evident in low-grade cervical intraepithelial neoplasia (CIN I: 66.67%), and showed a marked increase in high-grade lesions (CIN II: 87.5% and CIN III: 92%), reaching maximal levels in invasive carcinoma (100%) [Figure 3].

Both the proportion of positively stained cells and staining intensity demonstrated a significant linear trend with increasing lesion severity, indicating a strong ordinal association and progressive molecular alterations during cervical carcinogenesis.

[Figure 3] Linear trend of p16INK4a expression across the spectrum of cervical lesions. The bar chart demonstrates a progressive increase in p16INK4a positivity from benign lesions (0%) to low-grade

cervical intraepithelial neoplasia (CIN I: 66.67%), high-grade lesions (CIN II: 87.5% and CIN III: 92%), and invasive carcinoma (100%). This stepwise rise indicates a significant correlation between increasing p16INK4a expression and the severity of cervical epithelial dysplasia, supporting its role as a biomarker for disease progression.

Fisher's exact test: Furthermore, statistical evaluation using Fisher's exact test demonstrated a significant association between p16INK4a expression and lesion severity. Expression levels were significantly higher in neoplastic lesions compared to benign lesions ($p < 0.0001$). Furthermore, a statistically significant difference was observed between pre-neoplastic and neoplastic lesions ($p = 0.0336$), supporting the role of p16INK4a in distinguishing disease progression stages [Figure 4].

[Figure 4] Bar chart showing statistical significance of differences in p16INK4a expression between cervical lesion groups using Fisher's exact test. A highly significant difference was observed between benign and neoplastic lesions ($p < 0.0001$) and a significant difference between pre-neoplastic and neoplastic lesions ($p = 0.0336$). The y-axis represents −log10(p-value), with higher values indicating stronger significance. Data are based on 100 FFPE biopsy samples.

Diagnostic Performance: The diagnostic performance of p16INK4a showed a progressive increase in sensitivity with increasing lesion severity, while specificity remained consistently high (100%) across all categories.

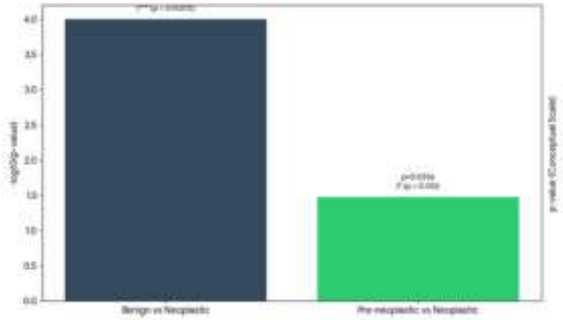

Figure 4:Showing significant differences in p16INK4a expression between lesion groups (Fisher's exact test), with strong significance for benign vs neoplastic ($p < 0.0001$) and moderate significance for pre-neoplastic vs neoplastic ($p = 0.0336$), based on 100 FFPE samples.

Table 5 Diagnostic Performance of p16INK4a Expression in Cervical Lesions

Diagnosis	Sensitivity	Specificity	PPV	NPV
CIN I	66.67%	100%	100%	90.48%
CIN II	87.50%	100%	100%	97.67%
CIN III	92.00%	100%	100%	97.40%
Invasive Carcinoma	100%	100%	100%	100%

These findings indicate excellent diagnostic accuracy of p16INK4a, particularly for high-grade lesions and invasive carcinoma.

Regression Analysis

Logistic regression analysis identified p16INK4a expression parameters, particularly staining intensity and the proportion of positive cells, as significant independent predictors of higher-grade cervical lesions and invasive carcinoma. Increased expression levels were significantly associated with a higher likelihood of disease progression. Among the evaluated variables, staining intensity emerged as the most robust predictor ($p < 0.05$), highlighting its prognostic significance in cervical neoplasia. Therefore, p16INK4a expression demonstrated a significant and progressive increase with advancing lesion severity. High-grade lesions and invasive carcinoma were characterised by strong and diffuse staining patterns, whereas low-grade lesions exhibited predominantly focal expression. A statistically significant correlation was observed between p16INK4a expression and histopathological grading. Furthermore, regression analysis confirmed p16INK4a as a reliable independent predictor of cervical neoplastic progression, supporting its utility in both diagnosis and prognostic assessment.

DISCUSSION

The present study evaluated the expression of p16INK4a across the spectrum of cervical lesions and demonstrated a significant correlation between biomarker expression and disease severity. The findings support the role of p16INK4a as a reliable diagnostic and prognostic marker in cervical carcinogenesis.[16,17]

A progressive increase in p16INK4a expression was observed from benign lesions to high-grade cervical intraepithelial neoplasia (CIN) and invasive carcinoma. All benign lesions were negative, whereas positivity increased from CIN I (66.6%) to CIN II (87.5%) and CIN III (92%), reaching 100% in invasive carcinoma. This stepwise pattern reflects the biological progression of HPV-mediated cervical neoplasia, where disruption of the retinoblastoma (Rb) pathway by high-risk HPV E7 oncoprotein leads to overexpression of p16INK4a (Sano et al., 1998; Klaes et al., 2001).[18,19]

The observed staining patterns further strengthened the diagnostic utility of p16INK4a. Low-grade lesions showed focal and patchy expression, whereas high-grade lesions and carcinomas demonstrated strong, diffuse, and continuous nuclear and cytoplasmic positivity. These findings are consistent with established literature, where block-type staining is considered a hallmark of transforming HPV

infections (Darragh et al., 2012; WHO Classification of Female Genital Tumours, 2020).[20,21]

Statistical analysis in the present study revealed a significant association between p16INK4a expression and lesion severity ($p < 0.0001$), with a clear distinction between benign, pre-neoplastic, and neoplastic lesions. Similar findings have been reported in recent studies, which emphasise the high diagnostic accuracy of p16INK4a in distinguishing high-grade lesions from reactive or benign conditions (Wentzensen et al., 2020; Schiffman et al., 2021).[22]

The diagnostic performance of p16INK4a in this study showed increasing sensitivity with lesion severity while maintaining 100% specificity across all categories. The sensitivity ranged from 66.67% in CIN I to 92% in CIN III, reaching 100% in invasive carcinoma. These findings are in agreement with recent meta-analyses demonstrating high specificity and variable sensitivity of p16INK4a, particularly in low-grade lesions (Yu et al., 2019; Jiang et al., 2022). The lower sensitivity in CIN I may be attributed to the transient nature of HPV infection and the absence of full oncogenic transformation in early lesions.[23]

Logistic regression analysis further identified staining intensity and percentage positivity as significant independent predictors of higher-grade lesions. Among these, staining intensity emerged as the most robust parameter, highlighting its importance in routine diagnostic practice. This observation aligns with recent studies suggesting that quantitative assessment of p16INK4a enhances diagnostic reproducibility and risk stratification (Ebisch et al., 2017; Clarke et al., 2022).

From a clinicopathological perspective, the study also demonstrated associations between lesion severity and factors such as age, parity, and clinical presentation. Higher-grade lesions and carcinoma were more common in multiparous women and in the fourth and fifth decades of life, which is consistent with known epidemiological patterns of cervical cancer (Arbyn et al., 2020). The findings of the present study reinforce the clinical utility of p16INK4a as an adjunct to histopathological evaluation. Its ability to differentiate between benign, low-grade, and high-grade lesions with high specificity makes it particularly valuable in borderline or ambiguous cases. Moreover, its strong association with disease progression supports its role in prognostic assessment and risk stratification.

CONCLUSION

The present study demonstrates a wide histopathological spectrum of cervical lesions, ranging from benign conditions to invasive carcinoma, with a notable predominance of high-grade lesions, particularly CIN III. This distribution reflects a considerable burden of advanced premalignant disease in the studied population.

The findings underscore the critical importance of early detection through effective cervical screening programs and prompt histopathological evaluation to enable accurate diagnosis and appropriate clinical management. Strengthening screening strategies and integrating adjunctive biomarkers such as p16INK4a may further enhance diagnostic precision and facilitate timely intervention, ultimately contributing to improved patient outcomes.

Limitations: The study is limited by its single-centre design and relatively small sample size, which may restrict generalizability. Lack of long-term follow-up data limits assessment of lesion progression or recurrence. Additionally, variability in sample adequacy and dependence on biopsy interpretation may introduce observational bias.

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