Original Article

Histopathological Evaluation of Cervical Biopsies with p16INK4a and Ki-67 Immunohistochemistry in Cervical Dysplasias

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Abstract

Background: Cervical cancer is a leading malignancy among women worldwide, strongly associated with persistent infection by high-risk human papillomavirus (HPV) types. Histopathology remains the diagnostic gold standard, but overlapping features between benign mimics and dysplastic lesions often pose challenges. Immunohistochemical (IHC) markers such as p16INK4a, a surrogate for HPV-mediated disruption of the Rb pathway, and Ki-67, a proliferation index marker, have emerged as useful adjuncts in diagnosis. **Material and Methods:** A prospective study was conducted on 60 cervical biopsies (age range: 20–75 years) over two years at the Department of Pathology, Government Medical College Kamareddy, Telangana. All cases underwent hematoxylin & eosin (H&E) examination, followed by IHC staining for p16INK4a and Ki-67 in 54 cases (8 negative for intraepithelial malignant lesion [NIML], 46 dysplasias). Expression patterns were graded, and statistical analysis was performed using Fisher's exact test. **Results:** Of the 60 biopsies, 13.3% were NIML, 76.7% dysplasias, and 10% malignancies. Among dysplasias, 47.8% were mild, 34.8% moderate, and 17.4% severe. p16 positivity rose progressively from 12.5% in NIML to 77% in mild, 94% in moderate, and 100% in severe dysplasias. Similarly, Ki-67 positivity increased from 25% (NIML) to 77% (mild), 81% (moderate), and 100% (severe). p16 showed higher sensitivity (86.9%) and specificity (87.5%) compared to Ki-67 (82.6% and 75%). Co-expression enhanced diagnostic confidence, particularly in high-grade lesions. **Conclusion:** Both p16INK4a and Ki-67 expression correlated significantly with increasing cervical intraepithelial neoplasia (CIN) severity. p16INK4a proved to be a highly sensitive and specific marker, while Ki-67 was a reliable proliferation marker. Their combined application can improve diagnostic accuracy, minimize inter-observer variability, and help differentiate true dysplasia from benign mimics.

Keywords: Cervical dysplasia, p16INK4a, Ki-67, immunohistochemistry, HPV, cervical cancer screening.

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Introduction

Cervical cancer is one of the most important public health problems affecting women worldwide. Globally, it is the fourth most common cancer in women, with an estimated 604,000 new cases and 342,000 deaths reported in 2020.[1] The burden of the disease is heavily skewed towards low- and middle-income countries, which account for nearly 90% of global deaths, largely due to inadequate screening and limited access to vaccination and treatment facilities.[2] In India, cervical cancer is the second most common malignancy in women, contributing to about one-fifth of the global mortality burden, with more than 120,000 new cases and nearly 77,000 deaths each year.[3] The disease most often affects women in their reproductive and perimenopausal years, producing a considerable socio-economic impact on families and communities.

Cervical carcinogenesis is a multistep process that usually develops from precursor lesions of the squamous epithelium, termed cervical intraepithelial neoplasia (CIN). These lesions arise within the transformation zone, where the squamocolumnar junction is particularly vulnerable to carcinogenic insults.[4] Persistent infection with high-risk human papillomavirus (HR-HPV), especially types 16 and 18, plays a central role in the pathogenesis of these lesions.[5] The viral oncogenes E6 and E7 promote tumorigenesis by inactivating p53 and retinoblastoma protein, resulting in uncontrolled cell proliferation and genomic instability.[6] Although HPV infection is very common in sexually active women, only a fraction progress to CIN and invasive carcinoma, with host factors such as immune competence, parity, smoking, and long-term hormonal contraceptive use acting as additional determinants.[7]

Cytological screening with the Papanicolaou test, introduced in the mid-twentieth century, has significantly reduced cervical cancer incidence and mortality in countries with organized screening programmes. However, the Pap test is hampered by modest sensitivity, subjective interpretation, and high rates of false negatives and false positives, necessitating repeated testing.[8] Histopathological evaluation of cervical biopsies

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remains the gold standard for diagnosis. Yet, even this is limited by inter- and intra-observer variability, particularly in differentiating reactive atypia, atrophy, and immature squamous metaplasia from true dysplasia.[9] Molecular assays for HPV DNA have high sensitivity and are increasingly used in primary screening, but they cannot distinguish transient infections from persistent infections that pose oncogenic risk.[10] These limitations highlight the need for complementary biomarkers that provide greater objectivity and reproducibility.

Immunohistochemistry offers such adjunctive tools. The cyclin-dependent kinase inhibitor p16INK4a is regarded as a surrogate marker for HR-HPV oncogenic activity. In normal cells, retinoblastoma protein regulates the expression of p16INK4a at low levels. When E7 oncoprotein inactivates retinoblastoma protein, there is compensatory overexpression of p16INK4a, detectable as diffuse nuclear and cytoplasmic staining in dysplastic and neoplastic cervical lesions.[11] Diffuse strong staining for p16INK4a correlates closely with high-grade lesions and is considered highly specific for HPV-related transformation.[12]

Ki-67 is another well-established marker of cellular proliferation. It is a nuclear protein expressed during all active cell cycle phases but absent in resting cells. In the normal cervix, Ki-67 expression is confined to basal and parabasal layers, whereas in dysplastic and neoplastic epithelium its expression extends to the middle and superficial layers.[13] Although Ki-67 lacks specificity when used alone, its value lies in reflecting abnormal proliferative activity, which correlates with lesion severity.[14]

Several studies have shown that using p16INK4a and Ki-67 enhances diagnostic accuracy, particularly in distinguishing high-grade CIN from benign mimics such as immature squamous metaplasia or reactive atypia. p16INK4a provides specificity for HPV-mediated transformation, while Ki-67 offers information about proliferative activity. Dual positivity strongly correlates with clinically significant disease and improves inter-observer agreement among pathologists.[15,16]

In view of the high burden of cervical cancer in India and the limitations of current diagnostic approaches, there is a clear need to integrate biomarker-based adjuncts into routine histopathological practice. The present study was undertaken to evaluate the histopathological features of cervical biopsies, to analyze the expression of p16INK4a and Ki-67 in cervical dysplasias, and to assess their correlation with the grade of lesion. The results are expected to enhance diagnostic accuracy, reduce misinterpretation, and improve clinical management of women with cervical lesions.

Materials and Methods

This prospective study was conducted in the Department of Pathology, Government Medical College Kamareddy, Telangana, over 24 months from July 2023 to June 2025. Written informed consent was obtained from all participants before the biopsy procedure, and confidentiality of patient identity was maintained throughout the study.

Study Design and Population: A total of sixty cervical biopsy specimens formed the study group, drawn from women who attended the outpatient and inpatient services of the Department of Gynaecology at GMC Kamareddy with symptoms suggestive of cervical disease. Selection was based on a clinical suspicion of underlying cervical pathology after detailed history and examination. The majority of these women presented with persistent or recurrent vaginal discharge that did not respond to routine treatment, a frequent sign of chronic cervicitis or early epithelial dysplasia. Others reported abnormal uterine bleeding patterns, including bleeding between menstrual cycles or spotting after sexual intercourse, which often indicate epithelial fragility and are recognised warning features for precancerous or neoplastic lesions. During per-speculum examination, several patients were noted to have an unhealthy cervix characterised by surface irregularity, congestion, erosions, contact bleeding, or suspicious growths, further strengthening the indication for tissue sampling. By focusing on this clinically high-risk cohort, the study ensured that the biopsies represented a spectrum of lesions in which histopathological evaluation and immunohistochemical analysis of p16INK4a and Ki-67 would yield maximum diagnostic and prognostic relevance.

Inclusion criteria

The prospective study included only those cervical biopsy specimens that fulfilled strict eligibility criteria to ensure accurate histopathological and immunohistochemical assessment. Women above 20 years of age were selected, as the transformation zone of the cervix becomes hormonally mature after adolescence and is the region most prone to persistent high-risk human papillomavirus infection and dysplastic change. Biopsies were required to demonstrate definite intraepithelial lesions—ranging from low-grade to high-grade squamous intraepithelial lesions—on initial hematoxylin and eosin examination so that the evaluation of p16INK4a and Ki-67 would specifically address lesions with potential for malignant progression and avoid confounding from purely inflammatory or benign reactive conditions. Only cases providing well-preserved and sufficiently large tissue fragments were accepted to allow routine paraffin embedding, serial sectioning, and consistent immunostaining, ensuring reliable antigen retrieval, reproducible grading, and meaningful statistical analysis. This carefully defined inclusion framework enhanced the quality and validity of the study findings by creating a uniform and clinically relevant sample for correlating histopathology with biomarker expression.

Exclusion criteria

In this study, biopsy specimens were carefully screened, and any samples that failed to meet strict quality requirements were excluded to maintain diagnostic accuracy and reproducibility of immunohistochemical analysis. Very small biopsies that were insufficient for complete paraffin block processing were not considered, as limited tissue hampers the preparation of multiple sections and often prevents representative evaluation of the entire epithelial architecture. Specimens exhibiting extensive necrosis, crush artefacts, or haemorrhage were also omitted because these changes can distort cellular and nuclear details, obscure the epithelial–stromal interface, and interfere with the interpretation of both routine hematoxylin and eosin

morphology and subsequent p16INK4a and Ki-67 immunostaining. Furthermore, women with a prior history of treated cervical malignancy, whether managed surgically, by radiotherapy, or by chemotherapy, were excluded since previous interventions can induce fibrosis, cytological atypia, and altered antigen expression, all of which may confound the grading of dysplasia and skew biomarker expression patterns. By enforcing these exclusion criteria, the study ensured that every case represented a primary, untreated lesion with well-preserved tissue, thereby guaranteeing the technical quality of histopathology, the reliability of immunohistochemical results, and the validity of the statistical correlations drawn between lesion grade and marker expression.

Sample Collection and Fixation: The gynecology team obtained all cervical biopsy specimens using strict aseptic techniques to minimise the risk of contamination and infection. Immediately after removal, each sample was immersed in an ample quantity of 10 % neutral buffered formalin, maintaining a minimum fixative-to-tissue volume ratio of about 10:1 to guarantee uniform penetration and adequate preservation. The tissues were left undisturbed in the fixative at room temperature for a period ranging from 12 to 24 hours, allowing complete cross-linking of proteins and stabilisation of cellular structures. This careful fixation protocol is critical because it halts autolytic changes, prevents bacterial overgrowth, and maintains the natural morphology of both nuclei and cytoplasm, which is indispensable for precise microscopic interpretation. Properly fixed specimens also retain antigenicity required for downstream immunohistochemical procedures such as p16INK4a and Ki-67 staining, ensuring consistent staining intensity and reproducible results throughout the histopathological and immunohistochemical evaluation.[17]

Tissue Processing and Embedding: After the fixation period, all cervical tissue samples underwent standard paraffin-embedding to obtain high-quality histological sections. The specimens were first dehydrated in a graded series of alcohol baths—progressing through 70%, 80%, 90%, and finally absolute alcohol—to remove water in a controlled manner and prevent tissue shrinkage or distortion. Following dehydration, the tissues were cleared in xylene, which effectively replaced the alcohol and created a medium compatible with paraffin infiltration. Each specimen was then saturated with molten paraffin wax at an appropriate temperature to ensure complete impregnation of all cellular and stromal elements. Paraffin blocks were cast in stainless-steel moulds and left to cool and harden at room temperature, producing firm blocks suitable for precise cutting. Thin sections measuring approximately 3–5 µm were subsequently cut on a rotary microtome, floated on a warm water bath to flatten the ribbons, and carefully transferred to clean glass slides coated with either egg albumin or silane. This coating provided an adhesive surface that firmly anchored the sections and prevented detachment during staining procedures, thereby preserving tissue integrity for routine hematoxylin and eosin examinations and immunohistochemical staining with p16INK4a and Ki-67.[17]

Hematoxylin and Eosin (H&E) Staining: All paraffin-embedded tissue sections were routinely stained with hematoxylin and eosin for detailed microscopic evaluation. The process began with deparaffinization in xylene, which completely removed the embedding wax and prepared the tissue for aqueous solutions. Slides were then rehydrated stepwise through descending alcohol concentrations to distilled water, ensuring uniform penetration of the stains. Nuclear staining was achieved with Harris hematoxylin, imparting a deep blue-violet colour to chromatin and nucleoli. Excess background was gently removed by brief differentiation in acid alcohol, followed by bluing in an alkaline solution to stabilize the hematoxylin–nucleic acid complex. The sections were then counterstained with eosin, which imparted varying shades of pink to cytoplasm, connective tissue, and extracellular components, providing a clear contrast to the nuclei. After staining, the slides were dehydrated through graded alcohols, cleared in xylene to render the tissue transparent, and finally mounted with a suitable medium under coverslips to preserve the preparation. This carefully executed routine H&E method produced crisp nuclear and cytoplasmic detail, which was essential for accurately identifying epithelial architecture, assessing dysplasia, and grading cervical intraepithelial neoplasia before immunohistochemical analysis with p16INK4a and Ki-67.[17]

Histopathological Classification: All stained sections were meticulously evaluated under the microscope by two experienced pathologists working independently to ensure that diagnostic interpretation remained objective and reproducible. This dual-review approach was deliberately adopted to minimize observer bias, a well-recognized source of variability in histopathological reporting, particularly when dealing with subtle or borderline epithelial alterations. Each biopsy was categorized according to the internationally accepted World Health Organization (WHO) histological classification of uterine cervix tumors, which provides standardized criteria for grading epithelial abnormalities and facilitates meaningful comparison with other published data. Within this framework, lesions exhibiting confined atypia limited to the lower one-third of the epithelium were designated as cervical intraepithelial neoplasia grade I (CIN I), synonymous with mild dysplasia or low-grade squamous intraepithelial lesion. Cases showing disordered maturation and nuclear atypia extending into the middle third of the epithelium were diagnosed as CIN II, equivalent to moderate dysplasia or high-grade squamous intraepithelial lesion. Biopsies displaying full-thickness epithelial immaturity, marked pleomorphism, and abnormal mitoses were classified as CIN III, corresponding to severe dysplasia or carcinoma in situ. In addition to these premalignant lesions, the examiners carefully documented other pathological findings of clinical relevance, such as chronic cervicitis with or without squamous metaplasia, reactive changes, nabothian cysts, and endocervical polyps. Recording these benign or inflammatory conditions was important because they can mimic dysplastic changes histologically and may influence patient management even in the absence of neoplasia. By adhering to a strict WHO-based diagnostic protocol and incorporating independent assessments, the study ensured high interobserver

agreement, precise lesion grading, and a comprehensive representation of both neoplastic and non-neoplastic cervical pathology, thereby strengthening the reliability of subsequent correlations with p16INK4a and Ki-67 immunohistochemical expression and the overall conclusions of the research.

Immunohistochemistry (IHC): Of the sixty cervical biopsy specimens processed during the study period, fifty-four fulfilled all quality requirements for immunohistochemical analysis and were therefore selected for biomarker evaluation. This subset included eight cases that were negative for intraepithelial malignant lesions (NIML) and forty-six cases diagnosed as various grades of cervical intraepithelial neoplasia (CIN), ensuring a representative spectrum of both non-neoplastic and dysplastic lesions for comparative assessment. Thin tissue ribbons measuring approximately 3–4 µm in thickness from each selected paraffin block were carefully sectioned using a precision rotary microtome to provide optimal morphology and antigen preservation. These sections were floated on a warm water bath to achieve uniform flattening and then transferred onto clean, silane-coated glass slides. The silane coating created a strong covalent bond between the glass surface and tissue proteins, greatly enhancing adhesion and preventing detachment during the multiple high-temperature and chemical processing steps required for p16INK4a and Ki-67 immunohistochemical staining. This meticulous preparation ensured that the antigenic epitopes remained intact, the staining was uniform and reproducible, and the slides could withstand repeated washing and incubation without tissue loss, thereby guaranteeing reliable interpretation of nuclear and cytoplasmic marker expression across the different categories of cervical lesions.

Antigen retrieval and staining protocol: Each selected section was first subjected to a carefully standardised sequence of preparatory steps for immunohistochemical staining to ensure optimal antigen exposure and consistent staining. Paraffin was completely removed by immersing the slides in fresh xylene baths, after which the tissue was gradually rehydrated through descending concentrations of alcohol to distilled water, restoring the native aqueous environment necessary for subsequent antigen retrieval and antibody binding. Heat-induced epitope retrieval was then carried out in a citrate buffer of pH 6.0 using a pressure cooker maintained at 120 °C for fifteen minutes, a critical step that breaks formalin-induced protein cross-links and unmasks target antigens without damaging cellular architecture. The sections were incubated with 3 % hydrogen peroxide for ten minutes to eliminate background interference from endogenous peroxidase activity. Non-specific antibody binding was minimized by applying the proprietary protein block supplied with the immunostaining kit, creating a uniform surface that enhanced the specificity of primary antibody attachment. After these preparatory steps, the slides were incubated overnight at 4 °C with the primary antibodies chosen for this study: mouse monoclonal anti-p16INK4a (clone G175-405, BioGenex, Fremont, CA, USA) and monoclonal anti-Ki-67 (clone BGX-27, BioGenex, Fremont, CA, USA). These antibodies were selected for their proven specificity and sensitivity in detecting cell cycle–related proteins associated with high-risk human papillomavirus–induced dysplasia. Following thorough rinsing in phosphate-buffered saline to remove unbound antibodies, the sections were treated with a secondary link antibody, which bridges the streptavidin–horseradish peroxidase complex. The chromogenic reaction was visualized using 3,3′-diaminobenzidine (DAB), producing a crisp brown precipitate at antigen–antibody binding sites and allowing clear microscopic identification of positive nuclear or cytoplasmic staining. Finally, the slides were counterstained with Mayer's hematoxylin to provide blue nuclear contrast, dehydrated, cleared, and mounted in DPX medium to preserve the tissue and staining quality. This meticulous stepwise protocol ensured high-resolution, reproducible detection of p16INK4a and Ki-67 expression, enabling accurate grading of cervical intraepithelial lesions and reliable comparison across all specimens.[19]

Positive controls were included for each marker: known squamous cell carcinoma sections for p16INK4a, and tonsillar tissue for Ki-67. Negative controls were processed simultaneously by omitting the primary antibody.

Interpretation of Immunostaining: Evaluation of p16INK4a immunostaining was carried out with a strict and reproducible protocol to ensure accurate assessment of biomarker expression. Sections were first screened for staining intensity and distribution within the squamous epithelium. Only those showing strong, continuous, diffuse nuclear and/or cytoplasmic staining were interpreted as positive, reflecting true overexpression of the p16INK4a protein associated with transforming high-risk human papillomavirus infection. In contrast, specimens exhibiting only faint or patchy cytoplasmic reactivity, or focal staining without clear nuclear involvement, were regarded as negative to avoid overestimating marker positivity due to background or non-specific uptake. The extent of staining was then quantified according to the well-established grading system proposed by Klaes and colleagues, which categorizes the proportion of positively stained epithelial cells into four levels: Grade 0 for less than 1 % positive cells, Grade I for 1–5 %, Grade II for 6–25 %, and Grade III for more than 25 %. This semi-quantitative scoring allowed consistent comparison between different cervical intraepithelial neoplasia grades and provided an objective measure of p16INK4a expression to correlate with histological severity and Ki-67 proliferation indices.[20] For Ki-67, nuclear staining alone was evaluated. Expression confined to basal or parabasal layers was regarded as physiological. Staining extending into suprabasal or superficial layers was considered abnormal and diagnostic of dysplasia. The labeling index (LI) was calculated as the number of positively stained nuclei per 100 cells under high-power magnification. Grading was done according to Shi et al.: Grade I (0–10%), Grade II (10–20%), and Grade III (>20%).[21]

Quality Control: All immunostained slides were examined independently by two qualified observers blinded to the patients' clinical details and preliminary histopathological diagnoses, ensuring an unbiased assessment of staining patterns and intensity. This double-masked evaluation minimized subjective variation and strengthened the reproducibility of the results. In instances where the two assessments differed—

whether in grading the proportion of positive cells or interpreting borderline staining—the slides were re-evaluated together in a joint session. This consensus review discussed microscopic fields in detail until a mutually agreed diagnosis and score were reached, eliminating inter-observer discrepancies. To further safeguard the technical validity of the procedure, internal tissue elements such as endocervical glands and stromal cells were monitored on every slide as intrinsic positive or negative controls. These internal reference structures confirmed the adequacy of antigen retrieval, staining uniformity, and reagent performance. This ensured that any lack of p16INK4a or Ki-67 expression within the epithelium reflected true biological absence rather than technical failure. This rigorous, multi-level quality control provided a reliable foundation for correlating immunohistochemical findings with histological grade and clinical relevance.

Statistical Analysis: All observations generated from the histopathological and immunohistochemical assessments were systematically entered into Microsoft Excel to create a master database, ensuring organised storage and easy retrieval of patient demographics, lesion categories, and biomarker scores. The compiled data were then subjected to statistical analysis using SPSS software version 20.0 (IBM Corp., Armonk, NY, USA), providing a robust platform for descriptive and inferential statistics. Basic distribution measures such as frequencies and percentages were calculated to summarise the prevalence of different histological diagnoses and the expression patterns of p16INK4a and Ki-67 across various grades of cervical intraepithelial neoplasia. To explore relationships between categorical variables—such as the association of marker positivity with lesion grade—Fisher's exact test was applied, an appropriate choice given the relatively small sample size and the presence of contingency tables with low expected cell counts. Diagnostic performance of each immunohistochemical marker was further evaluated by constructing 2×2 contingency tables to calculate sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV), providing a clear estimate of their ability to identify true-positive and true-negative cases. Throughout the analysis, a two-tailed p value of less than 0.05 was considered indicative of statistical significance, ensuring that the conclusions drawn about the diagnostic utility of p16INK4a and Ki-67 were supported by rigorous quantitative evidence.[22]

Results

The present investigation was conducted on sixty cervical biopsy specimens received and processed in the Department of Pathology, Government Medical College Kamareddy, Telangana, over 24 months from July 2023 to June 2025. The study group represented a wide reproductive and post-reproductive age spectrum, ranging from 20 to 75 years, with the mean age calculated to be approximately 45.8 years, reflecting the peak incidence of cervical epithelial abnormalities in middle-aged women. On routine histopathological evaluation, a diverse pattern of lesions was observed. Eight specimens (13.3 %) revealed only benign changes and were categorized as NIML, encompassing conditions such as chronic cervicitis, squamous metaplasia, and endocervical polyps. A significant majority of cases, forty-six biopsies (76.7 %), demonstrated features of epithelial dysplasia of varying severity—ranging from low-grade squamous intraepithelial lesions (CIN I) to high-grade lesions (CIN II and CIN III)—underscoring the high burden of preinvasive cervical disease in the study population. The remaining six cases (10 %) showed frank malignancy, predominantly squamous cell carcinoma, highlighting the malignant potential of untreated or progressive precursor lesions. For immunohistochemical assessment, fifty-four cases were selected, incorporating all eight NIML specimens and the entire group of dysplastic lesions. These sections were subjected to detailed staining and evaluation for the cell cycle–related biomarker p16INK4a and the proliferative marker Ki-67, allowing the study to correlate histopathological grading with molecular alterations and to determine the diagnostic and predictive value of these markers across the spectrum of benign, precancerous, and malignant cervical pathology.

Age Distribution: The age profile of the women in this study is presented in [Table 1] and reflects the typical epidemiological pattern of cervical epithelial disease. The largest cases occurred in the 40–49-year age bracket, accounting for 43.3 % of the cohort. This indicates that the fourth decade of life represents a critical period for cervical dysplastic and neoplastic changes. The next most common group comprised women aged 30–39 years, representing 23.4 % of the study population. This highlights that a substantial number of cases begin to emerge during the late reproductive years when hormonal influences and cumulative exposure to high-risk human papillomavirus infection contribute to disease progression. Fewer cases were recorded in women above 50 years, and the least representation was observed among those aged 20–29 years, with only a single case (1.6 %), suggesting that clinically significant cervical pathology requiring biopsy is relatively uncommon in early adulthood. This distribution emphasises the importance of targeted screening and early detection strategies in women beyond their third decade, as this is the period when the risk of high-grade precancerous lesions and invasive carcinoma begins to rise sharply [Figure 1].

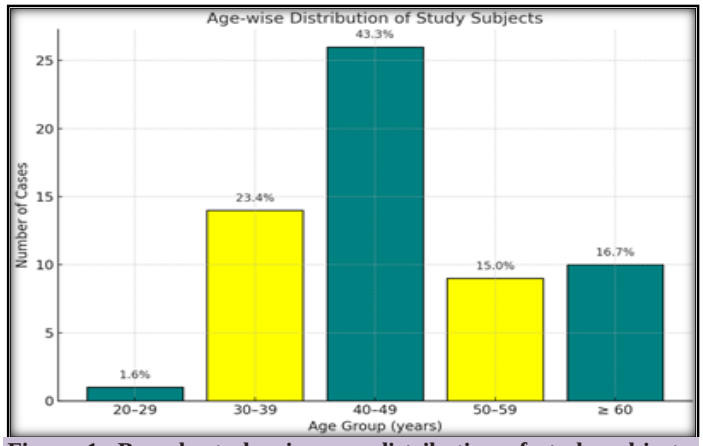

Figure 1: Bar chart showing age distribution of study subjects. Maximum cases were observed in the 40–49-year age group.

Spectrum of Histopathological Lesions

Histopathological evaluation of all sixty cervical biopsy specimens revealed that the overwhelming majority represented various grades of epithelial dysplasia, accounting for forty-six cases (76.7 %). This high proportion underscores the predominance of precancerous lesions within the study population. It highlights the crucial role of early detection and grading of cervical intraepithelial neoplasia in preventing malignant transformation. Benign, non-neoplastic conditions grouped as negative for intraepithelial malignant lesion (NIML) were identified in eight cases (13.3 %). These included entities such as chronic cervicitis, squamous metaplasia, and endocervical polyps, which, while not directly premalignant, can present clinically and microscopically with features that mimic dysplasia and therefore require careful distinction. Fully developed invasive malignancies were confirmed in six biopsies (10 %), predominantly comprising squamous cell carcinomas, reflecting the smaller but clinically significant subset of patients who had progressed beyond the intraepithelial stage. This distribution pattern—dominated by precancerous dysplastic changes with a smaller fraction of benign and frankly malignant lesions—illustrates the typical disease spectrum encountered in cervical pathology and underscores the importance of histopathology combined with adjunct immunohistochemistry for accurate diagnosis and timely intervention [Table 2, Figure 2].

Table 1: Age distribution of study subjects (n = 60)

Age group (years)	No. of cases	Percentage (%)
20–29	1	1.6
30–39	14	23.4
40–49	26	43.3
50–59	9	15.0
≥ 60	10	16.7
Total	**60**	**100**

Table 2: Distribution of Histopathological Lesions

Lesion type	No. of cases	Percentage (%)
NIML	8	13.3
Dysplasias	46	76.7
Malignancies	6	10.0
Total	60	100

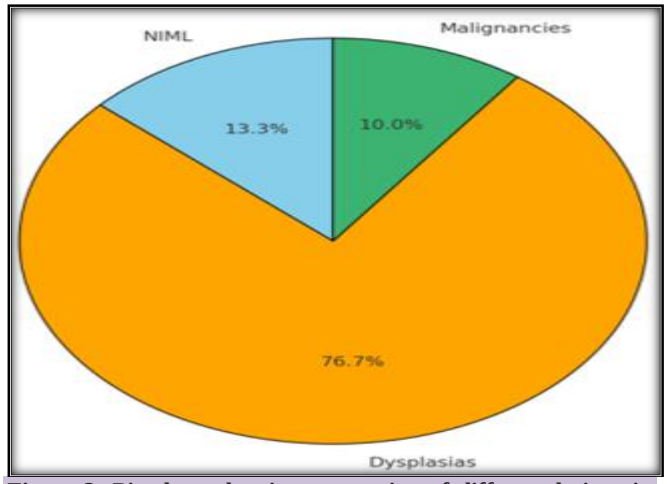

Figure 2: Pie chart showing proportion of different lesions in cervical biopsies

Subtypes of NIML: The group of cases classified as benign or negative for intraepithelial malignant lesion (NIML) comprised a variety of non-neoplastic conditions that can clinically mimic premalignant disease yet lack true dysplastic transformation. Within this category, chronic cervicitis was the most frequent finding, characterised by long-standing cervical stroma and epithelium inflammation, which can lead to epithelial hyperplasia and reactive atypia that may resemble low-grade dysplasia on routine examination. Some specimens also showed chronic cervicitis accompanied by squamous metaplasia or the presence of nabothian cysts—mucus-filled retention cysts that develop when squamous epithelium overgrows and obstructs endocervical glands—both of which can create irregular surface changes and raise clinical suspicion of pathology. Endocervical polyps formed another subset; these benign glandular proliferations present as polypoid growths and occasionally exhibit stromal edema or inflammation, which can be confused with neoplastic processes. Additionally, a few cases demonstrated koilocytic changes, reflecting human papillomavirus–related cytopathic effects without progression to intraepithelial neoplasia, and therefore were considered reactive rather than precancerous. Documenting these entities was important to highlight their potential to imitate dysplastic lesions and underscore the value of histopathology and adjunct immunohistochemistry in distinguishing reactive alterations from true cervical intraepithelial neoplasia [Table 3].

Table 3: Distribution of NIML cases (n = 8)

Lesion subtype	No. of cases	Percentage (%)
Chronic cervicitis	2	25.0
Chronic cervicitis with squamous metaplasia	1	12.5
Chronic cervicitis with nabothian cyst	1	12.5
Endocervical polyp	2	25.0

Koilocytic change	2	25.0
Total	8	100

Distribution of Dysplasias: Among the forty-six cervical biopsies diagnosed as dysplastic, the majority represented mild dysplasia (CIN I), which accounted for 47.8 % of the cases. Lesions in this category displayed architectural and cytological atypia confined to the lower one-third of the epithelium and correspond to low-grade squamous intraepithelial lesions that often arise from persistent human papillomavirus infection. Moderate dysplasia (CIN II) formed the next most common subgroup, comprising 34.8 % of dysplasias. It was characterised by disordered maturation and nuclear abnormalities extending into the middle third of the epithelial thickness, signifying a higher risk of progression. Severe dysplasia (CIN III), including carcinoma in situ with full-thickness epithelial atypia and numerous abnormal mitoses, accounted for the remaining 17.4 % of cases, representing the most advanced preinvasive stage before frank malignancy. This gradation reflects the natural history of cervical intraepithelial neoplasia and underscores the critical importance of early detection and timely intervention to halt progression from low-grade to high-grade lesions and ultimately to invasive carcinoma [Table 4, Figure 3].

Table 4: Distribution of dysplasia cases (n = 46)

Dysplasia type	No. of cases	Percentage (%)
Mild (CIN I)	22	47.8
Moderate (CIN II)	16	34.8
Severe (CIN III)	8	17.4
Total	46	100

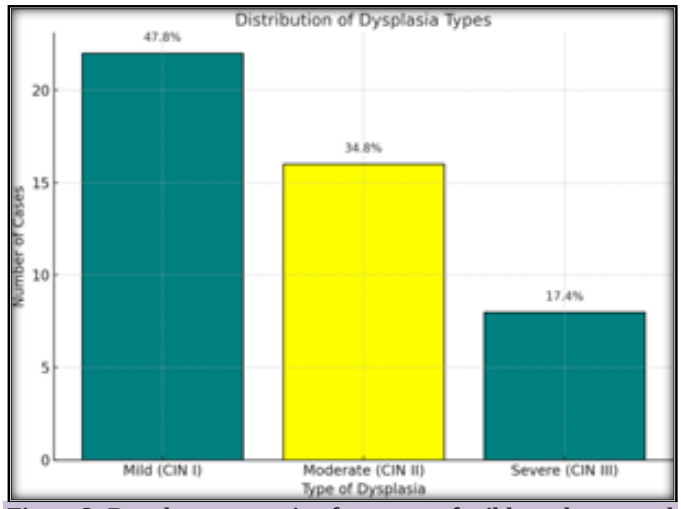

Figure 3: Bar chart comparing frequency of mild, moderate, and severe dysplasia.

Malignant Lesions: Within the malignant category of six cervical biopsies, squamous cell carcinoma not otherwise specified (SCC-NOS) was the predominant histological type, diagnosed in four cases and representing 66.7 percent of the malignancies. These tumors typically showed full-thickness epithelial atypia with invasive nests of malignant squamous cells breaching the basement membrane and extending into the underlying stroma, often accompanied by keratin pearl formation or intercellular bridges. These features characterise conventional invasive squamous carcinoma. The remaining two cases, accounting for 33.3 percent, were classified as microinvasive squamous cell carcinoma, an early invasive stage defined by very limited stromal penetration, usually less than 3 mm in depth and 7 mm in horizontal spread, and typically lacking significant lymphovascular involvement. Identification of microinvasive carcinoma is clinically important because the disease detected at this stage carries a much more favourable prognosis and can often be managed with conservative surgical approaches compared with overtly invasive tumors. This pattern reflects the gradual biological transition from high-grade intraepithelial lesions to early stromal invasion and then to established invasive carcinoma, emphasising the critical role of prompt histopathological diagnosis and appropriate immunohistochemical markers in planning timely and effective treatment.

p16INK4a Expression: The present study's immunohistochemical assessment of p16INK4a expression demonstrated a clear and consistent trend of rising positivity in parallel with the histological severity of cervical epithelial changes. Within the group classified as negative for intraepithelial malignant lesion (NIML), p16 positivity was rare, observed in only one case (12.5 percent). This finding indicates that strong and diffuse p16 expression is uncommon in non-neoplastic cervical conditions and supports the specificity of this marker for lesions linked to transforming human papillomavirus infection. In contrast, p16 expression increased markedly among dysplastic lesions. Of the twenty-two cases diagnosed as mild dysplasia (CIN I), seventeen (77 percent) exhibited positive nuclear and/or cytoplasmic staining, reflecting early but definite involvement of oncogenic HPV pathways in a significant proportion of low-grade lesions. The proportion of positive cases rose further with disease severity: fifteen of sixteen moderate dysplasia (CIN II) specimens, representing 94 percent, showed strong and diffuse staining, and all eight severe dysplasia (CIN III) specimens demonstrated unequivocal p16 positivity, amounting to 100 percent. This progressive stepwise rise from mild through moderate to severe dysplasia underscores the biological association between p16 overexpression and increasing disruption of cell-cycle regulatory mechanisms during neoplastic transformation. The data reinforce the diagnostic utility of p16 as a highly sensitive and specific biomarker for identifying and grading cervical intraepithelial neoplasia, distinguishing high-grade lesions that require active intervention from reactive or low-risk epithelial changes [Table 5, Figure 4].

Table 5: p16INK4a expression in NIML and dysplasias

Lesion type	Total cases	Positive	Negative	% Positive
NIML	8	1	7	12.5
Mild dysplasia	22	17	5	77.3
Moderate dysplasia	16	15	1	93.8
Severe dysplasia	8	8	0	100.0

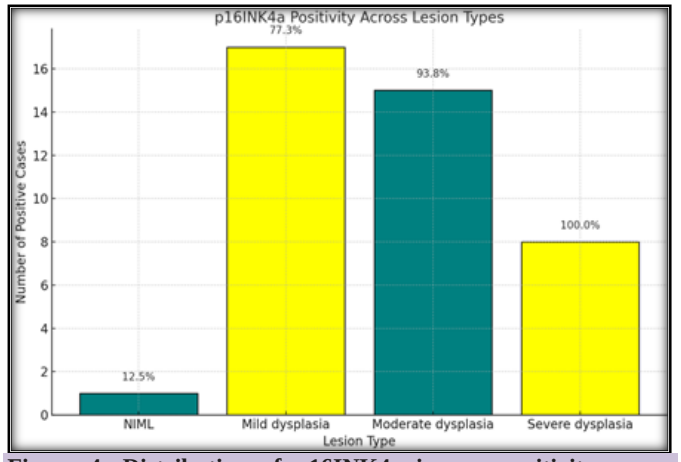

Figure 4: Distribution of p16INK4a immunopositivity across different cervical lesion categories. A clear stepwise increase in positivity is evident, from 12.5 % in negative for intraepithelial malignant lesion (NIML) to 77.3 % in mild dysplasia (CIN I), 93.8 % in moderate dysplasia (CIN II), and 100 % in severe dysplasia (CIN III), highlighting the strong correlation between p16 overexpression and increasing histological severity of cervical intraepithelial neoplasia

Ki-67 Expression: Ki-67 immunostaining in the present study revealed a pattern closely paralleling that of p16INK4a, with a progressive rise in expression corresponding to increasing histological severity of cervical lesions. In the negative for intraepithelial malignant lesion (NIML) group, only two of the eight cases (25 percent) exhibited Ki-67 positivity, and the staining was confined to the basal cell layer of the squamous epithelium. Such a basal-restricted pattern is generally considered a normal proliferative activity of the cervical epithelium and does not indicate neoplastic transformation. In contrast, the dysplastic categories demonstrated a marked expansion of Ki-67–positive nuclei beyond the basal zone. Among the twenty-two cases of mild dysplasia (CIN I), seventeen (77 percent) showed distinct nuclear staining extending into the epithelium's lower and occasionally middle third, signifying early but abnormal proliferative drive. Positivity increased further with disease severity: thirteen of sixteen moderate dysplasia cases (about 81 percent) showed Ki-67 expression reaching the upper epithelial layers, and all eight severe dysplasia (CIN III) specimens displayed diffuse and strong staining throughout the full epithelial thickness, amounting to 100 percent positivity. This stepwise extension of Ki-67 labelling from the basal compartment to the entire epithelial thickness underscores the close link between deregulated cell-cycle activity and the progression from low-grade to high-grade intraepithelial neoplasia [Table 6, Figure 5]. The findings reinforce the value of Ki-67 as a proliferation marker for distinguishing reactive epithelial changes from true dysplastic lesions and for grading cervical intraepithelial neoplasia in routine diagnostic practice.

Table 6: Ki-67 expression in NIML and dysplasias

Lesion type	Total cases	Positive	Negative	% Positive
NIML	8	2	6	25.0
Mild dysplasia	22	17	5	77.3
Moderate dysplasia	16	13	3	81.3
Severe dysplasia	8	8	0	100.0

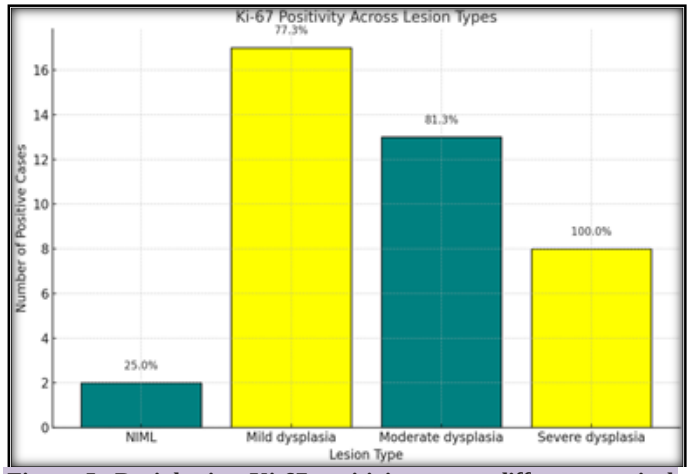

Figure 5: Deciphering Ki-67 positivity across different cervical lesion types.

Correlation of p16 and Ki-67: Analysis of immunohistochemical staining patterns revealed that both p16INK4a and Ki-67 showed a strong and statistically significant association with the histological grade of cervical disease. In low-grade squamous intraepithelial lesions (LSIL or CIN I), the proportion of positive cases for each marker was comparatively lower, consistent with the limited epithelial proliferation and lesser degree of cell-cycle deregulation typically seen in early dysplastic changes. In contrast, high-grade squamous intraepithelial lesions (HSIL, encompassing CIN II and CIN III) demonstrated a marked rise in expression of both markers, reflecting the more advanced disruption of cell-cycle control and the higher likelihood of integration of oncogenic human papillomavirus DNA. Importantly, when p16INK4a and Ki-67 were evaluated, their combined or co-expression provided greater diagnostic confidence than either marker alone. This was

especially evident in high-grade lesions, where simultaneous strong nuclear and/or cytoplasmic positivity for p16INK4a and extensive Ki-67 labeling across the epithelial thickness were almost uniformly present. Such dual positivity not only reinforces the diagnosis of HSIL but also helps distinguish true precancerous lesions from benign mimics or reactive epithelial changes, thereby supporting accurate grading and appropriate clinical management [Figure 6].

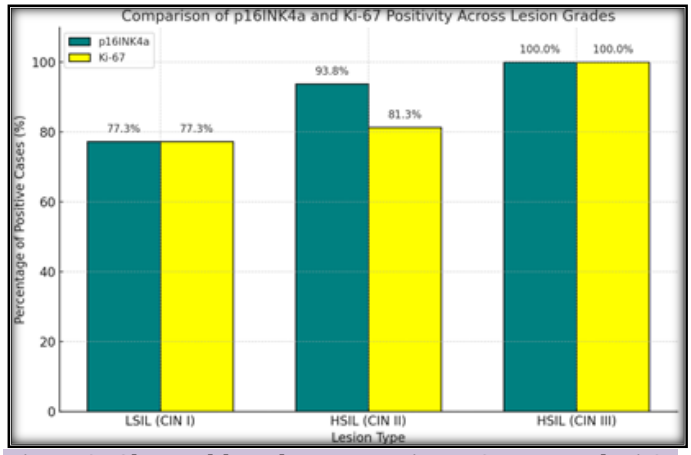

Figure 6: Clustered bar chart comparing p16INK4a and Ki-67 positivity across cervical lesion grades. It highlights that both markers show progressively higher positivity from LSIL (CIN I) to HSIL (CIN II and CIN III), with almost complete co-expression in severe dysplasia, supporting their combined diagnostic value in high-grade lesions

Diagnostic Performance and Accuracy of p16INK4a: A detailed statistical evaluation was carried out to determine the diagnostic accuracy of p16INK4a and Ki-67 in distinguishing dysplastic lesions (CIN I–III) from non-dysplastic cervical biopsies. The findings demonstrated that both markers possess strong diagnostic power, with p16INK4a showing slightly superior overall performance to Ki-67.

When all grades of cervical intraepithelial neoplasia were considered, p16INK4a immunostaining yielded a sensitivity of 86.9 % and a specificity of 87.5 %, indicating that this marker correctly identified the majority of true-positive dysplastic cases while rarely producing false positives among negative for intraepithelial malignant lesion (NIML) specimens. The positive predictive value (PPV) reached 97.5 %, meaning that nearly every case classified as positive was genuinely dysplastic. However, the negative predictive value (NPV) was lower at 53.8 %, reflecting that a negative p16INK4a result does not completely exclude the presence of dysplasia, especially in early or patchy lesions [Table 7].

Ki-67 also showed robust performance, though slightly less than p16INK4a. The sensitivity was 82.6 %, with a specificity of 75.0 %, signifying good ability to detect proliferative dysplastic epithelium but with a somewhat higher risk of non-specific positivity in reactive lesions. The PPV of 95 % indicates that most Ki-67 positive cases truly represented dysplasia, while the NPV of 42.9 % was lower, consistent with occasional false negatives when proliferative activity was limited to basal layers.

Fisher's exact test was applied to assess the strength of the association between biomarker expression and histological diagnosis because of the relatively small sample size and contingency tables with low expected counts. For both p16INK4a and Ki-67, p-values were less than 0.05, confirming a statistically significant difference in expression between dysplastic and non-dysplastic cervical tissues. These results establish that overexpression of either marker is strongly linked to the presence and severity of cervical intraepithelial neoplasia.

Table 7: Diagnostic performance of p16INK4a and Ki-67 in detecting cervical dysplasia

Marker	Sensitivity (%)	Specificity (%)	Positive Predictive Value (%)	Negative Predictive Value (%)	p value (Fisher's exact test)
p16INK4a	86.9	87.5	97.5	53.8	< 0.05
Ki-67	82.6	75.0	95.0	42.9	< 0.05

The combined statistical evidence and visual comparison underscore that p16INK4a is slightly superior to Ki-67 for both sensitivity and specificity, while Ki-67 remains a valuable adjunct to assess proliferative activity. Importantly, the significant p-values ($p < 0.05$) validate that expression of either marker is not random but closely related to the presence and grade of cervical intraepithelial neoplasia. These findings support the routine use of p16INK4a and Ki-67—particularly in combination—to strengthen diagnostic confidence and guide clinical management of cervical precancerous lesions.

Histopathological and Immunohistochemical Spectrum of Cervical Dysplasia

The progressive histological and biomarker changes observed across increasing grades of cervical intraepithelial neoplasia. In [Figure 7A], a hematoxylin and eosin–stained section at 40× magnification depicts mild dysplasia (CIN I), where nuclear enlargement and mild pleomorphism remain confined to the lower third of the squamous epithelium. [Figure 7B] shows the corresponding p16INK4a immunostain of this lesion, revealing only focal brown nuclear and cytoplasmic staining restricted to the basal and parabasal cells, a pattern consistent with early and limited viral oncogenic activity. In [Figure 7C], representing moderate dysplasia (CIN II), p16INK4a expression becomes diffuse and more intense, extending beyond the basal zone into the lower and middle epithelial layers, signifying greater disruption of cell-cycle control. [Figure 7D], an H&E section at 10×, demonstrates severe dysplasia (CIN III) with full-thickness epithelial atypia, loss of normal stratification, and numerous mitoses but no stromal invasion, fulfilling the criteria of carcinoma in situ. The corresponding p16INK4a immunostain in [Figure 7E] exhibits strong, block-like nuclear and cytoplasmic positivity involving the entire epithelial thickness, a hallmark of high-grade

squamous intraepithelial lesions driven by high-risk human papillomavirus. Finally, [Figure 7F] highlights the Ki-67 immunoprofile of the same high-grade lesion, where dense nuclear labeling extends throughout the full epithelial thickness, reflecting a marked proliferative drive and reinforcing the diagnosis of severe dysplasia. These images provide vivid morphological and molecular evidence of the stepwise escalation of disease and demonstrate how combined p16INK4a and Ki-67 staining greatly enhances diagnostic confidence in differentiating and grading cervical dysplastic lesions.

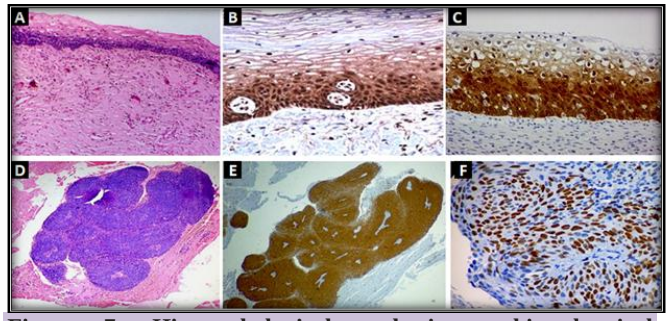

Figure 7: Histopathological and immunohistochemical spectrum of cervical intraepithelial neoplasia
(A) Mild dysplasia (CIN I) showing nuclear atypia limited to the lower one-third of the epithelium on H&E stain (40×).
(B) Mild dysplasia with focal basal and parabasal p16INK4a positivity (40×).
(C) Moderate dysplasia (CIN II) displaying diffuse p16INK4a nuclear and cytoplasmic staining extending into the middle third of the epithelium (40×).
(D) Severe dysplasia (CIN III/carcinoma in situ) on H&E stain showing full-thickness epithelial atypia without stromal invasion (10×).
(E) Severe dysplasia with strong block-type nuclear and cytoplasmic p16INK4a expression throughout the entire epithelial thickness (10×).
(F) Severe dysplasia with diffuse Ki-67 nuclear positivity across the full epithelial thickness, indicating high proliferative activity (10×).

Discussion

The present study provides a comprehensive histopathological and immunohistochemical evaluation of cervical biopsies, highlighting the diagnostic importance of p16INK4a and Ki-67 as reliable biomarkers for CIN. Sixty cervical biopsies were analysed over two years, with patient ages ranging from 20 to 75 years and a mean of 45.8 years. The highest proportion of cases occurred in the 40–49 year group, which corresponds to the peak period for high-grade cervical lesions and early invasive carcinoma in many population-based registries.[23] This distribution emphasises the need for targeted screening and early detection among women in their fourth and fifth decades of life.

A key observation was the progressive rise in p16INK4a expression with increasing lesion severity. Among negative intraepithelial malignant lesions (NIML) cases, only 12.5 % showed focal or weak staining, whereas positivity was recorded in 77 % of CIN I, 94 % of CIN II, and 100 % of CIN III lesions. This clear stepwise increase reflects the underlying biological changes driven by persistent infection with high-risk human papillomavirus (HPV) and the associated inactivation of the retinoblastoma (Rb) pathway.[24] The findings closely parallel earlier studies demonstrating that strong, diffuse nuclear and cytoplasmic p16 staining is a highly specific surrogate marker of transforming HPV infection.[25,26]

Ki-67 showed a similar but slightly different staining pattern. Basal-cell–restricted positivity was observed in only 25 % of NIML specimens, representing normal epithelial proliferation. In contrast, 77 % of CIN I, 81 % of CIN II, and all CIN III cases demonstrated suprabasal or full-thickness nuclear staining, reflecting increased and deregulated proliferative activity.[25] This stepwise upward expansion of Ki-67 expression mirrors the increasing grade of dysplasia and supports its role as a sensitive marker of abnormal cell-cycle activity and neoplastic progression.

The combined use of p16INK4a and Ki-67 further strengthened diagnostic confidence. Dual positivity was uncommon in low-grade lesions but nearly uniform in CIN II and CIN III, reinforcing their combined diagnostic value.[25,26] This is consistent with the Lower Anogenital Squamous Terminology (LAST) recommendations, which advocate p16 immunostaining for ambiguous or high-grade squamous intraepithelial lesions to improve diagnostic reproducibility.[24]

Statistical analysis confirmed these observations. Beyond Fisher's exact test, which revealed significant differences in marker expression between dysplastic and non-dysplastic tissues ($p < 0.05$), additional testing demonstrated a highly significant linear trend. A chi-square test for trend showed strong positive associations between lesion grade and biomarker positivity (p16INK4a $\chi^2=22.95$, $p=4.1\times10^{-5}$; Ki-67 $\chi^2=13.38$, $p=0.0039$), while Spearman's rank correlation demonstrated perfect positive correlations for both p16INK4a and Ki-67 ($\rho=1.0$, $p<0.001$), confirming that marker expression increases in an orderly fashion with advancing grade.

The diagnostic performance metrics observed in this study were also high. For p16INK4a, sensitivity and specificity were 86.9 % and 87.5 %, respectively, with a positive predictive value (PPV) of 97.5 % and a negative predictive value (NPV) of 53.8 %. Ki-67 achieved a sensitivity of 82.6 %, specificity of 75 %, PPV of 95 % and NPV of 42.9 %. These values agree with, or are slightly higher than, previously published series.[25,26] The very high PPVs for both markers indicate that positive staining highly predicts true dysplasia. At the same time, the relatively lower NPVs emphasise that negative staining cannot entirely rule out early or focal lesions and should always be interpreted alongside morphology.

From a clinical perspective, the combined use of p16INK4a and Ki-67 has significant implications for patient management. Accurate separation of low-grade squamous intraepithelial lesion (LSIL/CIN I) from high-grade intraepithelial lesion (HSIL/CIN II and CIN III) is essential for deciding between conservative monitoring and more aggressive excisional or ablative therapy. Histopathology alone can be limited by inter-observer variability, especially at the CIN I/II boundary, but the objective and reproducible staining patterns of these biomarkers help overcome such challenges.[24,25]

Well-defined inclusion and exclusion criteria, independent double-masked slide evaluation with consensus review,

validated antibody clones, and standardised immunohistochemical protocols with internal controls strengthen this study. Additional statistical tools, such as trend analysis and correlation tests, further support the conclusions. The limitations are the moderate sample size and the lack of long-term follow-up to document natural history or treatment outcomes. Larger multicentric studies incorporating HPV genotyping and longitudinal data will be useful to validate these markers' prognostic value further.

In conclusion, the present investigation demonstrates that p16INK4a and Ki-67 are reliable and complementary biomarkers for diagnosing and grading cervical intraepithelial neoplasia. Their strong, statistically significant association with lesion severity and high sensitivity, specificity, and predictive values support their routine use alongside histopathology. These findings agree with international guidelines and strengthen the case for integrating p16INK4a and Ki-67 testing into cervical cancer screening and early management strategies.[23–26]

Conclusion

This study demonstrates that p16INK4a and Ki-67 are highly effective adjuncts to routine histopathology in detecting and grading cervical intraepithelial neoplasia. Both markers showed a clear, statistically significant increase in expression from benign cervical tissue through CIN I and CIN II to CIN III, with near-uniform co-expression in high-grade lesions. p16INK4a exhibited slightly higher specificity and predictive value, while Ki-67 provided sensitive evidence of abnormal epithelial proliferation. Their combined use enhanced diagnostic confidence, reduced inter-observer variability, and provided strong support for accurate triage of patients requiring treatment. These findings align with international guidelines and underscore the importance of incorporating dual-marker immunohistochemistry into standard diagnostic practice for improved cervical cancer prevention and early intervention.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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