

DUAL BIOMARKER ASSESSMENT OF P16INK4A AND HSP27: ENHANCING CYTOHISTOLOGICAL DIAGNOSIS OF CERVICAL NEOPLASMS

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ABSTRACT

Background: Objective: Cervical cancer remains a major public health concern despite widespread cytological screening. Histopathology is the diagnostic gold standard, however, interpretation of premalignant and early invasive lesions can still be challenging. Biomarkers such as p16INK4a and Heat Shock Protein 27 (HSP27) may improve diagnostic accuracy of cervical neoplasms. This study was done to evaluate the cytohistological correlation of cervical lesions and assess the diagnostic utility of p16INK4a and HSP27 immunohistochemical expression in cervical neoplasms. **Materials and Methods:** This prospective study included 110 women who underwent conventional Papanicolaou smear and cervical biopsy over an 18-month period. Cytological findings were correlated with histopathological diagnosis. Immunohistochemical staining for p16INK4a and HSP27 was performed in 29 histologically confirmed neoplastic lesions, and staining intensity and distribution were analyzed. **Results:** The mean age of the study population was 44.2 ± 10.2 years. Histopathology revealed almost 72.7% of cases were chronic nonspecific cervicitis. Concordance between cytology and histopathology was highest for Adenocarcinoma (100%), Squamous cell carcinoma (75%) and Chronic cervicitis (81.3%) but lower for LSIL (33.3%), HSIL (20%). p16INK4a expression showed a progressive increase in intensity with increasing cervical intraepithelial neoplasia grade. Positivity increased from 61.5% in CIN I to 80.0% in CIN II and 100% in CIN III. HSP27 positivity was observed in 80.0% of premalignant lesions and 88.9% of malignant lesions. Concurrent expression of p16INK4a and HSP27 was identified in 60.0% of premalignant lesions and increased to 88.9% in malignant lesions. **Conclusion:** Cytohistological correlation remains essential in the evaluation of cervical lesions. The combined assessment of p16INK4a and HSP27 enhances diagnostic confidence, particularly in distinguishing high-grade cervical intraepithelial neoplasia from invasive cervical carcinoma, and serves as a valuable adjunct to routine histopathological evaluation.

INTRODUCTION

Cervical cancer is the fourth most frequently diagnosed cancer among women globally and remains a major public health concern despite advances in screening and vaccination. It ranks among the three most common cancers affecting women younger than 45 years of age. Approximately 97,000 new cases are diagnosed annually in India, highlighting the importance of improving early detection and diagnostic accuracy to reduce disease-related morbidity and mortality.^[1-4]

Persistent high-risk HPV infection is the primary cause of cervical carcinogenesis. Nearly all cervical squamous cell carcinomas and most adenocarcinomas are associated with HPV infection, while only a small proportion of squamous cell carcinomas are HPV-independent.^[2,5] The latest WHO classification recognizes HPV-associated and HPV-independent cervical squamous cell carcinomas as distinct entities. Although therapeutic management is similar for both subtypes, identifying HPV-associated tumors is important for disease prevention, screening

programs, prognostic assessment, and evaluating the impact of HPV vaccination.^[3-7]

The widespread implementation of cervical cytology-based screening programs has significantly reduced the incidence and mortality of cervical cancer through the early detection of precancerous lesions. Despite these advances, histopathological examination remains the gold standard for the definitive diagnosis of cervical lesions.^[4,8]

However, the histopathological interpretation of cervical intraepithelial neoplasia (CIN), particularly the distinction between low-grade and high-grade lesions and the identification of early stromal invasion, is often limited by interobserver variability. The incorporation of objective immunohistochemical biomarkers may improve diagnostic reproducibility and accuracy.^[5-9]

Among these biomarkers, p16INK4a is a well-established surrogate marker of high-risk HPV-induced oncogenic transformation and plays an important role in distinguishing high-grade squamous intraepithelial lesions from benign and low-grade lesions. In contrast, Heat Shock Protein 27 (HSP27), a molecular chaperone involved in cellular stress responses, has emerged as a promising biomarker with potential utility in differentiating intraepithelial lesions from microinvasive carcinoma.^[5,10]

Therefore, the present study was undertaken to evaluate the cytohistological correlation of cervical lesions and to investigate the diagnostic utility of p16INK4a and HSP27 immunohistochemical expression as complementary biomarkers for improving the diagnostic accuracy of cervical intraepithelial neoplasia and invasive cervical carcinoma.

MATERIALS AND METHODS

This prospective observational study was conducted in the Department of Pathology, Government Medical College over a period of 18 months. A total of 110 women who underwent both cervical cytological examination and cervical biopsy during the study period were included. Inadequate cervical biopsy specimens lacking ectocervical epithelium or containing only fibrocollagenous stromal tissue was excluded from the study. Cytohistological correlation was performed in all cases. Immunohistochemical analysis was carried out in histologically confirmed cases of cervical neoplasia. Conventional cervical smears were collected using Ayre's spatula and stained by the Papanicolaou staining technique.

Cervical biopsy specimens obtained by punch biopsy or cone biopsy were fixed in 10% neutral buffered formalin for 24–48 hours, processed routinely, embedded in paraffin wax, sectioned at 4–5 µm thickness, and stained with hematoxylin and eosin (H&E) for histopathological examination. Immunohistochemical (IHC) staining was performed on histologically suspected neoplastic lesions using the peroxidase-antiperoxidase (PAP) method. Tissue sections mounted on chrome alum-coated slides were deparaffinized, rehydrated, and subjected to heat-induced antigen retrieval using the pressure cooker method. The sections were subsequently incubated with monoclonal antibodies against p16INK4a and Heat Shock Protein 27 (HSP27). The percentage and intensity of immunoreactive cells were evaluated independently for each marker. p16INK4a expression was assessed using a combined intensity and proportion scoring system.^[6]

Intensity score

- **0: No staining**
- **1: Weak staining**
- **2: Moderate staining**
- **3: Strong staining**

Proportion score

- **0: No positive cells**
- **1: <1% positive cells**
- **2: 1–10% positive cells**
- **3: 11–33% positive cells**
- **4: 34–66% positive cells**
- **5: >66% positive cells**

The **total score** (0–8) was calculated by adding the intensity and proportion scores and categorized as:

- **0–2: Low expression**
- **3–5: Moderate expression**
- **6–8: Overexpression**

HSP27 expression was graded according to the percentage of positively stained cells;^[5]

- **0: 0% positive cells**
- **1: 1–29% positive cells**
- **2: 30–79% positive cells**
- **3: 80–100% positive cells**

Data were entered into Microsoft Excel and analyzed using IBM SPSS Statistics (Version 25.0). Descriptive statistics were expressed as frequencies, percentages, mean, and standard deviation where appropriate. Cytohistological correlation was assessed, and associations between biomarker expression and histopathological diagnosis were analyzed using the Chi-square test. A p-value <0.05 was considered statistically significant.

RESULTS

Table 1: Demographic details of study population

VARIABLE	CATEGORY	N (%)
Age	<30	10 (9.1)
	31-40	31 (28.2)
	41-50	47 (42.7)
	51-60	12 (10.9)

	>60	10 (9.1)
Leucorrhea	Present	60 (54.5)
Leucorrhea	Absent	50 (45.5)
VIA/VILI	Positive	72 (65.5)
VIA/VILI	Negative	38 (34.5)

Table 1: A total of 110 women were included in the study. The majority of participants belonged to the 41–50 years age group (47, 42.7%), followed by 31–40 years (31, 28.2%). Participants aged 51–60 years accounted for 10.9% (12), while those aged <30 years

and >60 years each constituted 9.1% (10) of the study population. Leucorrhea was present in 60 (54.5%) participants and absent in 50 (45.5%). On VIA/VILI screening, 72 (65.5%) participants tested positive, whereas 38 (34.5%) were negative.

Table 2: Pap smear–cervical biopsy correlation

Pap smear diagnosis	Pap n (%)	Biopsy outcome	Concordant n (%)	Discordant n (%)	Interpretation / reason for discordance
NILM / negative	91 (82.7%)	Chronic cervicitis/NILM: 74; CIN I: 11; CIN II–III: 4; SCC: 2	74 (81.3%)	17 (18.7%)	Focal/subtle CIN or occult carcinoma may be missed by cytology because of sampling limitation, inflammatory obscuration, low cellular yield or inadequate exfoliation.
LSIL	3 (2.7%)	Chronic cervicitis/NILM: 1; CIN I: 1; CIN II–III: 1	1 (33.3%)	2 (66.7%)	Discordance reflects either overestimation on cytology or upgrading on biopsy; small focal lesions and cytomorphological overlap may contribute.
HSIL	5 (4.5%)	CIN II–III: 1; SCC: 4	1 (20.0%)	4 (80.0%)	High-grade cytology may correspond to invasive SCC on biopsy; cytology cannot reliably establish stromal invasion.
Squamous cell carcinoma	4 (3.6%)	CIN II–III: 1; SCC: 3	3 (75.0%)	1 (25.0%)	One case showed CIN II–III on biopsy, possibly due to sampling of an intraepithelial component without the invasive focus.
Adenocarcinoma	1 (0.9%)	Adenocarcinoma: 1	1 (100%)	0 (0%)	Complete cytology–histology agreement.
Unsatisfactory Pap smear	6 (5.5%)	Chronic cervicitis/NILM: 2; CIN II–III: 3; adenocarcinoma: 1	Not assessable	Not assessable	Inadequate cellularity or obscuring blood prevents reliable cytologic interpretation; biopsy may subsequently reveal the underlying lesion.
TOTAL	110 (100%)	Chronic cervicitis/NILM: 77; CIN I: 12; CIN II–III: 10; SCC: 9; adenocarcinoma: 2	80/104 (76.9%)	24/104 (23.1%)	Overall concordance calculated among 104 interpretable Pap smears.

Note: Unsatisfactory Pap smears (n=6) are reported separately and excluded from the primary concordance calculation.

Table 2: 80/104 interpretable Pap–biopsy pairs were concordant (76.9%); 24/104 were discordant (23.1%). The 6 unsatisfactory smears were excluded from this denominator.

Cytology–histology correlation demonstrated an overall concordance of 76.9% among interpretable

Pap smears. Concordance was highest for adenocarcinoma (100%) and SCC (75.0%), while lower concordance was observed for LSIL (33.3%) and HSIL (20.0%). The principal discordant pattern was NILM on cytology with CIN I on biopsy.

Table 3: Immunohistochemical Expression of P16ink4a and Hsp27 According To Histopathological Diagnosis

Histopathological diagnosis	n	p16INK 4a Negative	p16INK 4a Low	p16INK 4a Moderate	p16INK4a Overexpression	HSP27 Negative	HSP27 Score 1	HSP27 Score 2	HSP27 Score 3	p16 + HSP27 co-expression
CIN I	13	5 (38.5)	4 (30.8)	4 (30.8)	0	4 (30.8)	3 (23.1)	6 (46.2)	0	6 (46.2)

CIN II	5	1 (20.0)	0	4 (80.0)	0	0	0	2 (40.0)	3 (60.0)	4 (80.0)
CIN III	2	0	0	1 (50.0)	1 (50.0)	0	0	1 (50.0)	1 (50.0)	2 (100)
Infiltrating adenocarcinoma	1	1 (100)	0	0	0	1 (100)	0	0	0	0
Mucinous adenocarcinoma	1	0	0	1 (100)	0	0	0	1 (100)	0	1 (100)
Squamous cell carcinoma	7	0	0	1 (14.3)	6 (85.7)	0	0	0	7 (100)	7 (100)
Total	29	7 (24.1)	4 (13.8)	11 (37.9)	7 (24.1)	5 (17.2)	3 (10.3)	10 (34.5)	11 (37.9)	20 (69.0)

Table 4: Comparison of P16ink4a and Hsp27 Expression between Premalignant and Malignant Lesions

Histopathological group	n	p16INK4a positive n (%)	p16INK4a moderate/overexpression n (%)	HSP27 positive n (%)	HSP27 score 2-3 n (%)	p16 + HSP27 co-expression n (%)
Premalignant lesions (CIN I-III)	20	14 (70.0)	10 (50.0)	16 (80.0)	13 (65.0)	12 (60.0)
Malignant lesions	9	8 (88.9)	8 (88.9)	8 (88.9)	8 (88.9)	8 (88.9)
Total	29	22 (75.9)	18 (62.1)	24 (82.8)	21 (72.4)	20 (69.0)

Tables 3 & 4: p16INK4a expression showed a progressive increase in intensity with increasing cervical intraepithelial neoplasia grade. Positivity increased from 61.5% in CIN I to 80.0% in CIN II and 100% in CIN III. Strong p16INK4a expression (moderate/overexpression) was observed in 50.0% of premalignant lesions compared with 88.9% of malignant lesions. HSP27 positivity was observed in 80.0% of premalignant lesions and 88.9% of malignant lesions. Concurrent expression of p16INK4a and HSP27 was identified in 60.0% of premalignant lesions and increased to 88.9% in malignant lesions. The highest degree of co-expression was observed in CIN III and squamous cell carcinoma.

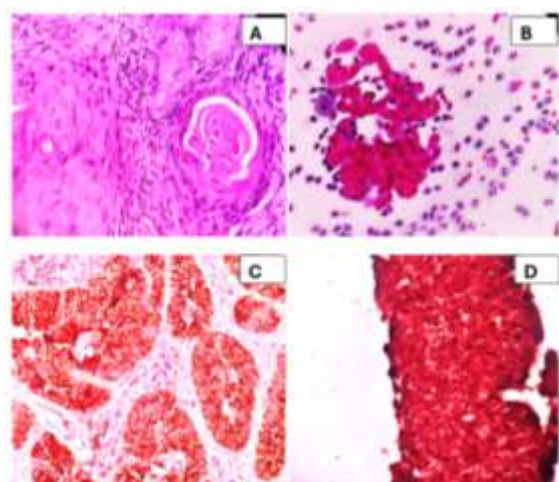


Figure 1 – A. Histopathological section of cervical biopsy depicting squamous cell carcinoma with keratin pearl formation (H&E, 40x) B. PAP smear depicting clusters of atypical endocervical cells. C. IHC of HSP27 showing diffuse cytoplasmic positivity D. IHC of p16INK4a showing block positivity

DISCUSSION

The accurate diagnosis and grading of cervical neoplastic lesions are paramount for effective patient management and reducing the global burden of cervical cancer.^[7] Although conventional Papanicolaou (Pap) smear cytology remains a cornerstone of cervical screening programs, its efficacy is frequently limited by low sensitivity and high interobserver variability.^[8-11] Histopathological evaluation via cervical biopsy serves as the diagnostic gold standard, yet it too faces challenges in distinguishing low-grade from high-grade intraepithelial lesions and identifying early microinvasion.^[10,12,13] The findings of the present prospective study underscore these diagnostic limitations and highlight the significant clinical utility of combining p16INK4a and Heat Shock Protein 27 (HSP27) as an immunohistochemical adjunct panel.

In our study of 110 participants 42.7% of our study population were of 41-50 years of age. CIN was the most common diagnosis between the age group of 45-80 years of age, which was similar to the study done by Pradhan B. Mital V P,^[14] and Choudhary et al.^[15]

Leucorrhoea was found to be the main symptom in these cases which correlates with the findings of Saha et al,^[16] Zainab S. Nayani et al,^[17] whose study showed an incidence of leucorrhea in 46.87% of cases with CIN lesions.

In our study cohort of 110 patients, the highest concordance between cytology and histopathology was observed in benign/inflammatory lesions, specifically NILM/chronic cervicitis (85.7%). The concordance rates for LSIL/CIN I and HSIL/CIN II-III were notably low at 8% and 16.6%, respectively. This substantial rate of cytohistological discrepancy aligns with existing literature given by Sung et al⁽¹⁸⁾, Srivatsav et,^[19] al which frequently documents the diagnostic gap between exfoliative cytology and tissue biopsy.

The present study demonstrated a good cytohistological correlation between Pap smear and cervical biopsy findings, particularly in cases negative for squamous intraepithelial lesions (NILM). Of the 110 cases evaluated, 91 were reported as negative for intraepithelial lesions on Pap smear. Histopathological examination of these cases revealed chronic cervicitis in 80 cases, while the remaining cases showed CIN I, resulting in a false-negative rate of 12.9%.

However, discrepancies were observed in higher-grade lesions and invasive malignancies. Among the cases diagnosed as CIN II on cervical biopsy, 2 cases (33.3%) were missed on Pap smear due to unsatisfactory cytological samples. Similarly, 11.9% of infiltrating adenocarcinoma cases were not detected on cytology and were identified only on subsequent histopathological examination of cervical biopsy specimens. These findings underscore the inherent limitations of Pap smear, particularly in the evaluation of glandular lesions and in samples with inadequate cellularity.

Of the five cases reported as high-grade squamous intraepithelial lesion (HSIL) on cytology, four cases (80%) were confirmed as squamous cell carcinoma (SCC) on histopathology, while one case was ultimately diagnosed as chronic cervicitis. The latter likely represented an overdiagnosis attributable to inflammatory atypia, highlighting the potential for reactive cellular changes to mimic high-grade lesions on cytological assessment. Notably, all cases diagnosed as SCC on Pap smear showed complete concordance with cervical biopsy findings.

These observations are comparable to those reported by Zainab S. Nayani et al,^[17] who found that cytohistological correlation was relatively poor for low-grade CIN lesions but improved significantly for CIN II and CIN III lesions. In contrast, the present study demonstrated a relatively low false-negative rate of 12.9%. Furthermore, 14.7% of cases were categorized as HSIL on cytology and subsequently confirmed as SCC on histopathology. Despite its limitations, Pap smear remains a valuable, cost-effective, and minimally invasive outpatient screening tool. When used in conjunction with cervical biopsy, it significantly enhances the diagnostic accuracy and early detection of cervical neoplasms.^[20,21]

As detailed in our results, the primary drivers of this discordance include technical and sampling limitations, such as low cellular yield, obscuring inflammation or blood, and the relative inability of conventional Pap smears to adequately capture endocervical or highly focal lesions.^[12,22,23]

Furthermore, histopathology identified a higher proportion of advanced premalignant and invasive lesions compared to cytology, confirming that while Pap smears excel as a low-cost screening tool, they cannot substitute for histopathology when definitive management decisions are required.^[10,26] To improve diagnostic reproducibility, we evaluated the expression of p16INK4a, a well-established

surrogate marker of high-risk human papillomavirus (hr-HPV) oncogenic activity.^[11,28-29] In a healthy cell cycle, p16INK4a acts as a tumor suppressor that inhibits cyclin-dependent kinases. However, persistent infection with hr-HPV leads to the expression of the E7 oncoprotein, which binds and degrades the Retinoblastoma (Rb) protein.^[10,12] Nayar et al,^[30] stated that the functional loss of Rb eliminates its negative feedback on p16INK4a transcription, resulting in a marked reciprocal overexpression of p16INK4a.^[12,14] In our study, p16INK4a positivity was identified in 75.9% of neoplastic lesions, with 62% of cases exhibiting moderate-to-strong overexpression. This high level of expression was strongly associated with high-grade cervical intraepithelial neoplasia (CIN II/III) and invasive carcinomas, compared to low-grade changes.^[12,14] Consequently, p16INK4a serves as an objective tool to clarify ambiguous morphological features and confirm HPV-driven oncogenic transformation.^[11,14] While p16INK4a is highly sensitive for HPV-mediated cell cycle disruption, it cannot reliably differentiate between high-grade precancerous lesions and true invasive carcinomas since both display robust staining.^[12,14] To address this limitation, our study incorporated Heat Shock Protein 27 (HSP27) into the biomarker panel. HSP27 is a low-molecular-weight molecular chaperone that is induced under cellular stress and plays a crucial role in regulating apoptosis, maintaining cytoskeletal integrity, and facilitating cell survival.^[13] Our findings revealed that HSP27 positivity matched p16INK4a at 75.9%, with a prominent concentration of high scores (scores 2 and 3 accounting for 72.4% of cases) seen in advanced CIN and invasive squamous cell carcinomas. The upregulation of HSP27 reflects the increased metabolic stress and altered apoptotic pathways characteristic of malignant progression and stromal invasion.^[13] By assessing HSP27 alongside p16INK4a, pathologists can better characterize the transition from a confined intraepithelial lesion to invasive disease, particularly when early microinvasion is histologically ambiguous.^[13] The combined evaluation of p16INK4a and HSP27 provides an objective, reproducible, and biopathologically logical diagnostic strategy. While p16INK4a successfully marks the onset of HPV-induced dysplastic transformation,^[12] HSP27 aids in risk stratification by tracking aggressive cellular stress responses associated with tumor invasion.^[13] Integrating this dual-marker panel into the diagnostic workflow significantly reduces interobserver variability, provides greater confidence in grading borderline biopsies, and enhances the overall accuracy of cytohistological evaluations.^[12,13,14] This integrated approach is crucial for preventing both the overtreatment of low-grade lesions and the dangerous undertreatment of occult high-grade malignancies. While p16INK4a effectively serves as a surrogate marker for high-risk HPV-induced oncogenic transformation and dysplastic progression, HSP27

acts as a crucial indicator of cellular stress responses associated with advanced malignant progression and tumor invasion. Taken together, this dual-biomarker panel enhances diagnostic confidence, helps reduce interobserver variability, and improves risk stratification, particularly in distinguishing high-grade cervical intraepithelial neoplasia from invasive cervical carcinoma.

CONCLUSION

The study demonstrates that cytohistological correlation remains an essential component in the comprehensive evaluation of cervical lesions, helping to identify diagnostic gaps caused by the limited sensitivity of conventional cytology in precursor and malignant lesions. The combined assessment of p16INK4a and HSP27 provides a valuable, objective, and complementary diagnostic strategy in evaluating cervical lesions. Integrating these biomarkers into routine evaluation serves as a reliable adjunct to histopathology, optimizing clinical decision-making and patient management.

Declaration of Generative AI in Scientific Writing

The authors declare that no generative AI or AI-assisted technologies were used in the writing process of this manuscript.

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Author Contributions

Conceptualization: Monisha G, Dhanalakshmi S, Birundha B.

Methodology: Monisha G, Dhanalakshmi S, Birundha B

Investigation: Monisha G, Dhanalakshmi S, Writing – Original Draft Preparation: Monisha G, Dhanalakshmi S, Birundha B ; Writing – Review & Editing: Monisha G, Dhanalakshmi S, Birundha B. All authors have read and agreed to the published version of the manuscript.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Research Ethics and Patient Consent

All procedures performed in the current study were approved by IRB and/or national research ethics committee (reference number and date) in accordance with the World Medical Association Declaration of Helsinki. IEC.NO – 42/2020 from institutional ethical committee. Informed consent was obtained from all individual participants included in the study.

Conflict of Interest:

The authors declare no conflict of interest.

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