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Pathology

IMMUNOHISTOCHEMICAL EVALUATION OF P16 AND KI-67 IN PREMALIGNANT AND MALIGNANT LESIONS OF UTERINE CERVIX

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ABSTRACT **Introduction** - Cervical cancer remains a significant public health concern worldwide, with premalignant lesions progressing to invasive carcinoma over time. The biomarkers P16 and Ki-67 have emerged as valuable tools for identifying and characterizing these lesions due to their roles in cell cycle regulation and proliferation. **Objectives -** This study aimed to investigate the expression patterns of p16 and Ki67 in premalignant and malignant cervical lesions. Specific objectives included categorizing the lesions as HPV Associated and HPV Independent on the basis of p16 immunoexpression and to compare their histomorphological correlation. Study also has an objective to find out the dual positivity of p16 and Ki67 in premalignant and malignant lesions of cervix to improve the diagnostic accuracy of cervical lesions. **Methods-** A retrospective observational study was conducted involving histopathological samples from patients diagnosed with various stages of cervical lesions. Immunohistochemical staining for p16 and Ki67 was performed, and semi-quantitative method was used for scoring. These were then analyzed using statistical methods. **Results-** Results showed that p16 expression was positive in 71.42 % of premalignant and 98.61 % of malignant cases. Ki67 positivity was noted in 85.71 % of premalignant and 88.88 % of malignant cases. Dual positivity was found in 71.42% of premalignant and 88.88% of malignant cases. HPV Association was found in 98.61 % of malignant lesions and HPV Independent lesions were found to be 1.39 %. There was no significant correlation found in histomorphology of HPV associated or HPV Independent lesions. **Conclusion-** It was concluded that p16 and Ki67 positivity increased when the grade of lesion increased from premalignancy to malignancy and that dual positivity of these biomarkers could help in increasing the diagnostic accuracy of cervical lesions.

KEYWORDS : P16 , Ki-67 , HPV.

INTRODUCTION

In the world, cervical cancer ranks fourth in terms of frequency among women; in 2022, there were about 6,60 ,000 new cases and 3,50, 000 deaths due to cervical cancer.[1] Low- and middle-income nations have the greatest incidence and fatality rates from cervical cancer. This reflects significant disparities caused by social and economic factors, as well as limited access to National HPV vaccination, cervical screening, and treatment facilities.[2] The Main cause of cervical cancer is the human papilloma virus. Cervical cancer is six times more common in women living with HIV than in those without the virus.[3]

Cervical cancer can be effectively prevented and is a highly cost-effective strategy when combined with screening for pre-cancerous lesions and a prophylactic HPV vaccination.[4]

P16 expresses in individual epithelial cells of the lower genital tract.[5] The expression level of P16 is higher in cells of high-grade precancerous and cancerous cervical lesions.[6,7] When P16 is lost in malignant lesions due to malignant transformation it may serve as a diagnostic tool. It may also serve as a prognostic tool when it is overexpressed due to malignant transformation and pRb failure. Therefore, in order to use P16 immunohistochemistry as a diagnostic or prognostic tool, it may be important to examine the expression of P16 in human tumours.

The expression of P16 has to be understood in order to create novel anticancer medications that function to re-establish P16 as a key tumour suppressor.[8] When determining the effectiveness of cervical cancer screening techniques, immunohistochemistry was deemed to be the gold standard.[9]

Monitoring P16 expression is one of the most economical methods of diagnosing HR-HPV because of its overexpression in cervical malignant tissue. Therefore, it is possible to use P16 overexpression as a surrogate biomarker for high-risk HPV infection in cervical cancer.[10]

The nuclear and nucleolar protein Ki-67 is exclusively expressed during the G1, S, G2, and M stages of the cell cycle and not during G0 and early G1 phases. High cellular proliferation is correlated with overexpression of Ki-67. Enhanced Ki-67 staining may be a sign of HPV infection because HPV infection causes enhanced epithelial cell proliferation in infected tissue. When the standard hematoxylin and eosin stain of tissue is unable to distinguish between a low-grade lesion and a high-grade lesion due to reactive changes, immature metaplasia, or atrophic changes of the cervix, the investigation of these two molecular biomarkers may prove helpful. Our research sought to assess the expression of P16 and Ki-67 in CIN1, CIN2/3, and invasive carcinoma.[11]

P16 And Ki 67

- P16 Expression: P16 expression is usually upregulated in precancerous lesions (such as Cervical Intraepithelial Neoplasia, or CIN) and cervical cancer.[12]
- This is often due to the integration of high-risk Human Papillomavirus (HPV) into the host genome, leading to increased expression of P16 as a part of the host defense mechanism against viral oncoproteins.[13]
- Ki-67 Expression: High Ki-67 expression indicates increased proliferation activity within the cervical epithelial cells. In precancerous lesions, a higher Ki-67 index (percentage of positively stained cells) suggests a higher risk of progression to invasive cancer.[14]
- Combined Interpretation: When both P16 and Ki-67 are highly expressed, it suggests ongoing abnormal proliferation of cervical cells, which is a hallmark of precancerous and cancerous states. This dual positivity serves as a strong indicator of the presence and severity of cervical lesions.[15]

Aim And Objectives

- **Aim-**
- To evaluate the immunohistochemical expression of P16 and Ki-67 in premalignant and malignant lesions of uterine cervix.

Objectives-

1) To assess the histomorphological spectrum of premalignant and malignant lesions of the cervix.

2) To evaluate P16 and Ki-67 expression in premalignant and malignant lesions of the uterine cervix.
3) To assess the morphology of P16 positive and P16 negative premalignant and malignant epithelial neoplasms of the uterine cervix.

Research Methodology
Methodology :
Study/ Research Design - Cross Sectional Study

Sample Selection –
Inclusion Criteria
1) Histopathologically confirmed cases of premalignant lesions i.e. LSIL and HSIL and cervical carcinoma cases.
2) Tissue adequate for further processing and immunohistochemical application.

Exclusion Criteria
1) Inadequate and autolysed tissue.
2) Infective and inflammatory disorders of cervical specimen.

Data Collection: The data, including the patient's age and histopathological data, were obtained from the pathology records of the Department of Pathology, S.N. Medical College, Agra.

Sample Size Calculation – Convenient sampling method was followed. A total of 79 samples were then analyzed for P16 and Ki-67 immunostaining.

Study Method And Tools -
Processing Of Specimen:
Cervical biopsy specimens and hysterectomy specimens received were fixed in 10% formal saline and processed routinely and immunohistochemical procedure was then performed using P16 and ki67 antibodies.

Scoring Of Ki-67:
Grading Of Ki-67 Expression:
According to Alshenawy H. et al.[16], grades 1+, 2+, and 3+ were assigned when the Ki-67 index was less than 5%, 5–30%, and more than 30%, respectively, based on the observation of 200 epithelial cell nuclei spread throughout the entire epithelial layer in a high-power field as shown in the table 1. A grade of 0 was assigned for cells showing no positivity.

P16 scoring –
For the quantitative evaluation of P16 staining, the percentage of positive cells was measured and then classified according to both nuclear and cytoplasmic staining (Block positivity). Immunoreactivity to P16 was classified into three groups according to the percentage of stained cells; weak, variable and strong corresponding to less than 5% of the cells, 5%-50% of the cells (containing weak and strong areas of intensity), and more than 50% of the cells stained for P16, respectively.

The Allred score was calculated by measuring the percent of stained cells scored as 0 to 8 (Proportionate Score) and Intensity score (weak, intermediate, and strong) (Table 1 and 2). [17]

Table-1: Proportionate Score and Intensity Score

Proportion Score (PS)		**Intensity Score (IS)**	
Value	**Significance**	**Value**	**Significance**
0	None	0	None
1	<1%	1	Weak <5%
2	1-10%	2	Intermediate 5-50%
3	10-33%	3	Strong >50%
4	33-66%		
5	>66%		

Table-2: Allred Score = (PS) + (IS)

ALLRED SCORE	GRADING
0	0
1	2,3,4
2	5,6
3	7,8

The possible values of Allred score are 0 – Allred 0; 1 – Allred 2, 3, 4; 2 – Allred 5, 6; 3 – Allred 7, 8 (Allred score 1 is not possible).[17]

Ethical Considerations – Approval for the study was obtained from the Institutional Ethics Committee before the commencement of study. Informed consent was obtained from the patients.

Data Collection Procedure – Data was collected from the histopathology lab of the Pathology Department.

Plan For Data Analysis –The correlation between two variables was assessed using the Spearman correlation coefficient. p value of less than 0.05 was considered to be statistically significant.

RESULTS –
Table-3: Distribution of cases according to P16 expression and type of lesion. (N=79)

Lesion	P16 expression (Allred score)				p-value
	Negative	Weak	Moderate	Strong	
SCC	1 (1.47%)	0 (0%)	10 (14.7%)	57 (83.8%)	<0.001
Adeno	0 (0%)	0 (0%)	1 (25%)	3 (75%)	
HSIL	0 (0%)	0 (0%)	3 (60%)	2 (40%)	
LSIL	2 (100%)	0 (0%)	0 (0%)	0 (0%)	

Correlation score = 0.536 p-value = <0.001

Table-4: Distribution of cases according to Ki-67 expression and type of lesion. (N = 79)

Lesion	Ki-67 expression				p-value
	Grade 0	Grade 1	Grade 2	Grade 3	
SCC	1 (1.47%)	7 (10.3%)	27 (39.7%)	33 (48.53%)	0.002
Adeno	0	0	1 (25%)	3 (75%)	
HSIL	0	0	3 (60%)	2 (40%)	
LSIL	1 (50%)	1 (50%)	0	0	

Correlation score = 0.209 p-value = 0.064

Table-5: Distribution of cases according to status of lesion and dual positivity. (N = 79)

Dual positivity (Both P16 and KI-67)	Pre-malignant	Malignant	p-value
Positive	5 (6.32%)	64 (81.01%)	0.185
Negative	2 (2.5%)	8 (10.1%)	

Table-6: Association of HPV with category of lesion

CATEGORY OF LESION	HPV ASSOCIATED (P16 POSITIVE)	HPV INDEPENDENT (P16 NEGATIVE)
LSIL	0%	100%
HSIL	100%	0%
SQUAMOUS CELL CARCINOMA	98.53 %	1.47%
ADENOCARCINOMA	100%	0%
ADENOSQUAMOUS CARCINOMA	100 %	0%

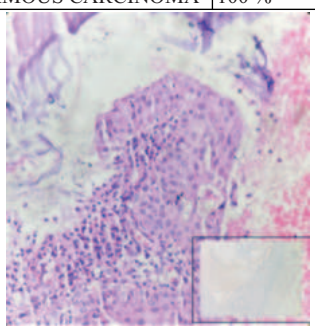

Figure-1: Low grade squamous intraepithelial neoplasm (LSIL) H&E stain (Objective 40X) and P16 IHC negative expression (inset)

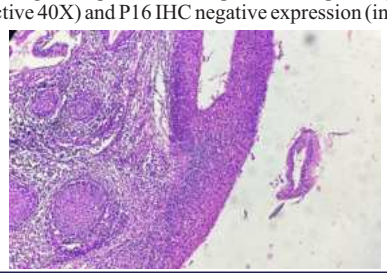

Figure-2: High grade intraepithelial neoplasm (HSIL). H&E (Objective 40X)

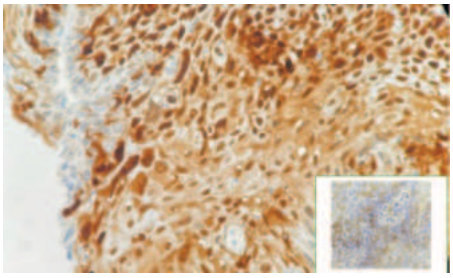

Figure-3: P16 expression and Ki67 (inset) IHC Positivity in case of HSIL (Objective 40X)

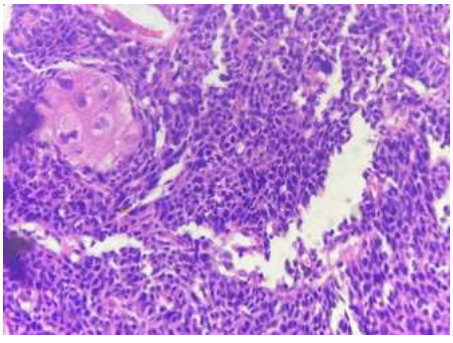

Figure-4: Keratinizing squamous cell carcinoma H&E (Objective 40X)

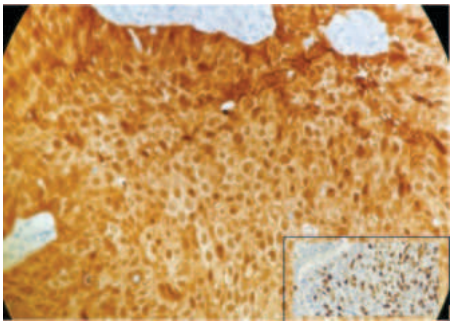

Figure-5: Keratinizing SCC showing P16 expression and Ki67 expression (inset) (Objective 40X)

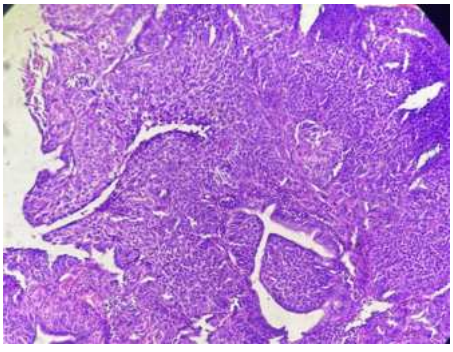

Figure-6: Non- Keratinizing squamous cell carcinoma H&E (Objective 10X)

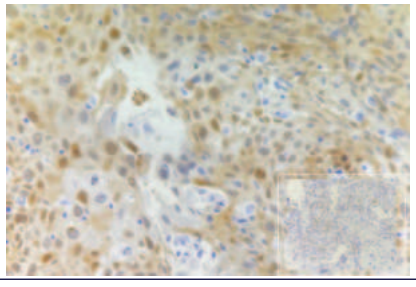

Figure-7: NKSCC showing P16 expression and Ki67 expression (inset) (Objective 40X)

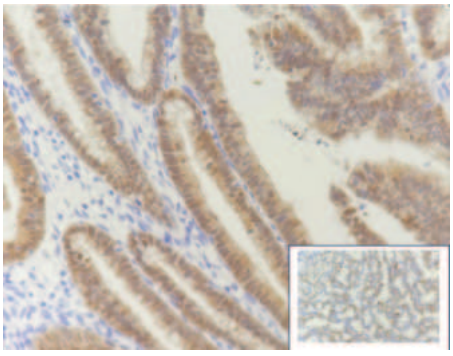

Figure-8: Adenocarcinoma showing P16 expression and Ki67 expression (inset) (Objective 10X)

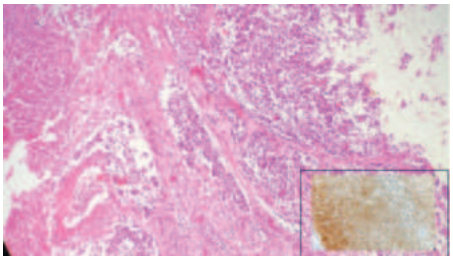

Figure-9: Adenosquamous carcinoma cervix H&E (Objective 40X) and P16 expression (inset)

- P16 expression was positive in 71.42 % of premalignant and 98.61 % of malignant cases.
- Ki67 positivity was noted in 85.71 % of premalignant and 88.88 % of malignant cases.
- Dual positivity of P16 and Ki-67 biomarkers was found in 71.42% of premalignant and 88.88% of malignant cases.
- Considering P16 as a surrogate marker for HPV, P16 positive lesions were considered as HPV Associated and P16 negative lesions were considered HPV Independent.
- HPV Association was observed in 98.61 % of malignant lesions and HPV Independent lesions were found to be 1.39 %.
- There was no significant correlation found in histomorphology of lesions and HPV association , in our study.

DISCUSSION -

- Age group of patients in the present study ranged from 30-80 years. Mean age of the study group was 55.25 years.
- A total of 8.83% of premalignant cases and 91.13 % of malignant cases were analyzed.
- Out of which seven cases were diagnosed with squamous intraepithelial lesions – two cases were diagnosed with LSIL, while five cases were diagnosed with HSIL.
- It was observed that 96% of cases had positive P16 expression while 87% of cases had a positive Ki-67 expression.
- The present study showed the positivity of P16 as 50 % in LSIL cases and of 100 % in HSIL cases. Malignant lesions showed positivity of 98.6 %, out of which SCC showed positivity of 98.53 % and adenocarcinoma cases showed 100% P16 positivity.
- One case of adenosquamous carcinoma was included in our study which showed P16 positivity.
- Our study showed 71.42 % Ki-67 positivity in premalignant lesions and 88.88% positivity in malignant lesions. In premalignant lesions, LSIL showed positivity of 50% and HSIL showed positivity of 100%.
- Additionally, dual positivity of P16 and Ki-67 was found in 87.3% of cases.

The age group of patients in our study correlated with study done by R.K. et al[18] and showed an elderly population of females with cervical preneoplastic and neoplastic abnormalities.

Other studies done by Devi N. et al[19], Sarwath H. et al[20], Ghosh A. et al[21], etc. showed a majority of patients in an age group that was a decade lower than our study and this could be because of the lesser number of premalignant cases included in our study. Moreover, it

could also be due to less awareness in the population and hence presentation occurs at late ages.

The present study showed positivity of P16 as 0 % in LSIL cases and of 100 % in HSIL cases. Malignant lesions showed positivity of 98.53 %, out of which SCC showed positivity of 98.53% and adenocarcinoma cases showed 100% P16 positivity.

The study done by Kanthiya K. et al[13], showed P16 positivity of 10.4% in LSIL cases which was close to our study.

The study by Lesnikova I.et al[22] showed P16 positivity of CIN I as 72.3%. the study by Bharadwaj V. et al[23] showed P16 positivity of 40% in LSIL cases. The study by Hebbar A. et al[24] showed P16 positivity of 50% in CIN I cases.

These results were not concordant with the results of the present study. This discrepancy could be due to the smaller number of LSIL cases included in our study.

HSIL cases showed 100% of P16 positivity in our study which was then compared with findings of previous studies.

The study done by Lesnikova I.et al[22] showed P16 positivity of 91% in CIN II cases and of 98.3% in CIN III cases which was in close approximation with our study.

Study by Priyadarshani R.et al[25] showed that 100% of low grade lesions showed negative P16 staining and all the cervical carcinoma patients showed P16 positivity. Thereby concluding "that as the grade of cervical neoplasia grew, so did the expression of P16. Therefore, P16 could be helpful as an adjunct in histology sections to find HPV in these lesions."[25]

In comparison, our study showed positivity of 71.4 % in premalignant lesions of cervix.

These findings again showed a wide difference which can be rectified by using a larger sample size and by doing HPV testing along with the P16 correlation.

Our study used a semi-quantitative method of evaluating P16 expression and it concluded that on assessing the intensity score of P16, both grade 2 and grade 3 intensities were equally expressed in HSIL lesions.

In our study, we found out that 98.16 % of malignant lesions were P16 positive.

These findings were similar to studies done by Qin S. et al[26] (95.65%), Sangwaiya A.et al[27] (95.2%), Hebbar A. et al[24] (100%) and Kanthiya K. et al[10] (91.3%).

Our study showed that 1.84 % of malignant lesions were negative for P16 and were classified as HPV Independent.

The study R.K. et al[18] and Vedula B.et al[23] which showed that 10.7% and 11.91% of malignant lesions were negative for P16.
Hence, through different studies it was proved that HPV Independent lesions were also reported and there is hence a need to classify and report them separately.

In the present study, 96.2 % of total malignant cases were identified as HPV Associated, out of which 3.2 % of SCCs were found to be HPV Independent. Adenosquamous carcinoma in our study showed P16 positivity and was hence classified as HPV Associated.

Thereby proving that as the grade of lesion increases from premalignant to malignant, we have seen an increasing positivity rate of P16 signifying HPV association.

Our study shows 71.42 % Ki-67 positivity in premalignant lesions and 88.88% positivity in malignant lesions. In premalignant lesions, LSIL showed positivity of 50% and HSIL showed positivity of 100%.

A study done by Kanthiya K. et al[10] showed 22.6% positivity in LSIL cases whereas study done by Hebbar A.et al[24] showed 70% positivity in CIN I lesions.

Similarly HSIL cases have shown 100% positivity in our study. Out of which 60% showed grade 2 positivity and 40% showed grade 3 positivity resulting in overall positivity of 100%, as none of the lesion was negative for Ki-67.

The study done by Kanthiya K. et al[10] showed 75.4% positivity in HSIL lesions and study by Hebbar A. et al[24] have shown 90% and 100% positivity in CIN II and CIN III lesions respectively. The study by Hebbar A. et al[24] showed concordant results with our study.

The study by Devaki P. et al[28] showed that the most common result of Ki-67 immunostaining was 3+ in cases of stage III (81%), poorly differentiated carcinomas (76.2%), and keratinizing SCC (75.6%) i.e as the grade of lesion increased, so the Ki-67 positivity.

The studies by Shi Q. et al[26], Sangwaiya A. et al[27], Sarma U. et al[29] have shown positivity of 41.9%, 96.5% and 18.03% respectively. Our study has shown Ki 67 positivity of 71.42% in premalignant lesions.

Hence, it was observed that there was an increasing rate of positivity from LSIL to carcinoma in all the mentioned studies, and the same trend was noted in our study as well. It was because Ki-67 is a proliferative marker and shows maximum positivity in malignant lesions.

Our study has classified lesions as HPV Associated and HPV Independent on the basis of P16 positivity. 98.6% of malignant lesions were HPV Associated and 1.4 % of malignant lesions were found to be HPV Independent.

When we categorized the lesions according to their morphological diagnosis, it was found that 100 % of KSCC s were HPV Associated and 97% of NKSCCs were HPV Associated.

There was only one case in our study which was found to be HPV Independent and its morphological diagnosis was Non-Keratinizing SCC.

All the adenocarcinomas in our study were found to be HPV Associated.

One case of adenosquamous carcinoma was also included in our study and it was found to be HPV Associated.

It can be said through our findings that HPV Independent malignancies predominantly show non keratinizing pattern of SCC but since only single case proved to be HPV Independent in our study, we cannot make out a conclusion about the morphological correlation of HPV.

Moreover 97% of Non-keratinizing SCCs were HPV Associated similar to Keratinizing SCCs, we would like to conclude that there was no significant correlation between HPV positivity and morphological diagnosis of malignant lesions.

Dual positivity of P16 and Ki-67 was calculated in our study and it showed positivity of 71.42 % in premalignant conditions and 88.88 % in malignant conditions.

Our study comprised of 91% malignant cases and only 9% of premalignant ones indicating the need for improvement in the cervical screening methods in our area, hence helping in early diagnosis of premalignant conditions.

According to WHO 2020 classification, considering P16 as a surrogate marker for HPV infection, histopathological diagnosis of premalignant and malignant lesions in our study were classified as HPV Associated and HPV Independent lesions.

100% of LSILs were HPV Independent, 100% of HSILs were HPV Associated, 98.53% of SCCs were HPV Associated,1.47% of SCCs were HPV Independent and 100 % of adenocarcinomas and adenosquamous carcinomas were HPV Associated.

Limitations Of Our Study –

Our study comprised of a small sample size which was the major limitation of our study. Moreover, HPV testing along with P16 immunoexpression would enhance the diagnostic accuracy.

CONCLUSION

In current study, we have concluded that -

- P16 expression was negative in low grade intraepithelial lesions of cervix, it was found to be 100% positive in High grade intraepithelial lesions though grading of positivity was variable between grade 2 and grade 3.
- We also found out that P16 immunoexpression was positive in 98.61 % of malignant cases.
- SCCs were 98.53 % positive for P16 immunoexpression and 1.47 % were negative for P16 immunoexpression.
- Adenocarcinomas were 100% positive for P16 immuno-expression.
- Hence we concluded that P16 immunoexpression was found to be increasing as the grade of lesion changed from premalignant to malignant.
- Single case of adenosquamous carcinoma in our study was also found to be positive for P16 immunoexpression.
- Further in our study, we classified the lesions as HPV Associated and HPV Independent on the basis of P16 positivity according to the WHO classification.
- It was found that 98.61 % of lesions were HPV Associated and 1.38 % of lesions were HPV Independent.

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