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ROLE OF P16 AS A POTENTIAL BIOMARKER FOR CERVICAL LESIONS

Pathology

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ABSTRACT

Introduction: Cervical cancer is fourth most common cancers affecting women worldwide. Human papilloma virus is prime risk factor. In HPV infection, the viral gene E7 binds to RB protein and functionally inactivates it which causes release of E2F. Accumulation of E2F increases p16 transcription. Thus p16 overexpression will allow to identify dysplastic lesions. **Objectives:** To diagnose cervical lesion by histopathological examination and study p16 expression in premalignant and malignant lesions of cervix. **Materials And Methods:** Six month prospective study between Jan2022 – June 2022. This Study included cervical biopsies. On hematoxylin and eosin stained slides histological classification and immunohistochemical analysis was done. Cases are scored as positive when staining was expressed in either cytoplasm or both nuclei and cytoplasm. Scoring is done based on percentage positivity of tumor cells and staining intensity. **Observation And Result:** Among 25 cases taken for IHC staining using p16INK4a antibody which were CIN1, CIN2, CIN3, Squamous cell carcinoma, and total of 18 cases were positive. Among positive cases of CIN as level of dysplasia increases there is increase in intensity of immunostaining. **Conclusion:** Expression of p16INK4a provide a surrogate marker for persistent HPV infection.

KEYWORDS

p16, Squamous cell carcinoma, CIN- Cervical intraepithelial lesion, HPV- Human papilloma virus.

INTRODUCTION

Cervical cancer is fourth most common cancer in women. 604000 new cases and 342000 deaths were estimated globally in 2020.[1]

Numerous studies have demonstrated that human papillomavirus (HPV) infection is a key factor in the development of cervical cancer. In fact, practically all preneoplastic and neoplastic lesions of the cervix have been found to be infected with HPV.[2]

Papilloma virus is a double-stranded DNA virus encased in a 72-sided icosahedral protein capsid. There are more than 120 different forms of HPV, which can be classified as high-risk, intermediate-risk, and low-risk. The persistent high-risk type HPV16 and 18 cause infection of the cervical epithelium which appears to trigger neoplastic progression.[3]

Since over 80% of low-grade squamous intraepithelial lesions (LSIL) spontaneously disappear and about 10% advance to high-grade squamous intraepithelial lesions (HSIL) or cervical cancer, LSIL/CIN1 are often monitored without treatment.

CIN grades 2 and 3 are collectively classified as HSIL and excisional treatment is the standard way to manage these lesions though their progression rates to cancer are around 20% and 40% respectively. [4]

Since Pap smear tests have limited utility due to the substantial number of false positive results and the high inter-observer variability of smear interpretation, this provides compelling evidence that a more sensitive and precise test is required for enhancing cervical cancer screening and precisely detecting precancerous lesions.

HPV infects immature basal cells of squamous epithelium in areas of breaks or immature metaplastic squamous cells at squamocolumnar junction. HPV has to induce DNA synthesis in host cells so it has to reactivate mitotic cycle in such cells. HPV activates cell cycle by interfering with Rb and p53. HPV contributes to neoplastic progression through two viral oncoproteins E6 and E7 which interact with various cell cycle regulatory proteins.

The protein p16INK4a (henceforth referred to as p16) is a cellular protein involved in cell cycle regulation, and is strictly regulated in normal cells. Due to the transforming activity of the E7 oncogene of all high-risk human papillomavirus (HR-HPV) types, p16 is strongly overexpressed in dysplastic cervical cells and may be easily detected by immunohistochemistry (IHC).

P16 is a cyclin-dependent kinase (CDK)-4 inhibitor and specifically binds to cyclin D–CDK4/6 complexes to regulate the cell cycle at the G1-S interphase. P16 is integral to p-retinoblastoma (p-Rb) mediated control of the G1-S phase transition of the cell cycle; it puts a brake on the cell cycle by inactivating the CDKs that phosphorylate Rb protein. In pre-neoplastic and neoplastic cervical lesions associated with high risk HPV infection, there is functional inactivation of Rb by HPV E7 protein. This results in an accumulation of p16 protein, because normally Rb inhibits transcription of p16. P16 expression can also be regarded as a marker of E7 gene activity. Therefore, p16 may be considered a surrogate marker for the activated oncogene expression of HR-HPV in dysplastic cervical cells. [4,5]

Currently, p16 staining is advised to assist in cases where there is a dispute over the morphological interpretation of a sample to help distinguish between low-grade and precancerous disease. Thus, the objective of this study was to evaluate the usefulness of p16 immunohistochemical staining in cervical specimen interpretation.

AIM:

To diagnose cervical lesion by histopathological examination and to study p16 expression in premalignant and malignant lesions of cervix.

MATERIALS AND METHODS:

This prospective observational study comprises of 25 cases of cervical lesions from Jan 2022 to June 2022 which were collected from the department.

Histopathology:

Tissue was preserved in formalin and processed in routine paraffin embedded sections for histopathological report. Histological classification was done on hematoxylin and eosin stained slides according to WHO criteria. Proliferating cells restricted to the lower third of the epithelium characterized as LSIL/CIN1, one-third to two-thirds dysplastic cells described as HSIL/CIN2, and HSIL/CIN3 referred to full-thickness dysplasia.

Immunohistochemistry:

Sections with thickness of 3-4 microns were taken on slides coated with adhesive. Tissue sections were deparaffinized using xylene and dehydrated using graded alcohols. Then rehydration with water, Antigen retrieval done in Multi Epitope Retrieval System using Tris EDTA buffer of ph 9. Blocking endogenous peroxidase activity by 0.3% hydrogen peroxide for 5to10 mins. The sections were incubated with p16ink4a primary mouse monoclonal antibody. (Biogenex company) as per the instructions of the manufacturer. The poly excel M/R poly HRP (non-biotin, micropolymer based) and diaminobenzidine (DAB) detection system procedure was followed. Counterstaining was done with gills hematoxylin. Production of brown product was considered as positive.

Cases of known squamous cell carcinoma of cervix served as positive control slides. Positivity in stromal fibroblasts serve as an internal positive control.

Evaluation of p16ink4a immunostaining:
Cases were scored as positive when staining was expressed in either cytoplasm or both nuclei and cytoplasm. Scoring is done based on percentage positivity of tumor cells and staining intensity.

Scoring of percentage positive tumor cells was carried out as follow;
0% staining as negative
1-5% as 1+
5-25% as 2+
>25% as 3+
Intensity of immunostaining was taken as weak, moderate and strong intensity.

Patterns of p16ink4a staining:
- Negative, if there was no positive cells or <1% of cells were positive.
- Patchy if focally aggregated cells involving <25% of area of epithelium were positive.
- Diffuse basal if positive cells lay in continuity with each other in lower half of a broad area of epithelium.
- Diffuse full thickness, if positive cells lay in continuity with each other and involve full thickness of a broad area of epithelium.

Statistical analysis:
The statistical significance of associations between the various qualitative parameters was evaluated using Chi-square test. Statistical significance was taken at p value of <0.05.

RESULTS:
Total cases studied were 25, their clinical details pertaining to presenting complaints were obtained. Most of the patients presented with complaints of white discharge constituting about 40 %(Table1).

Table 1 : Distribution of cases based on major presenting complaint		
Clinical symptoms	No. of cases	Percentage
White discharge per vagina	10	40%
Bleeding per vagina	07	28%
Post coital bleeding	05	20%
Pain in abdomen	03	12%
Total	25	100%

In our study among 25 cases taken which were CIN 1, CIN 2, CIN 3, Squamous cell carcinoma, Majority of patients were in age group of 35 to 60 years. In our study least reported cases were that of microinvasive carcinoma, adenocarcinoma and adenosquamous carcinoma. Most common age group affected among CIN1and CIN2 was 31-40 years. Among CIN3 most affected was 41-50 years and SCC was common in 41-50 and 51-60 years. (Table 2).

Table 2 : Distribution of cases according to age				
Age in years	CIN1	CIN2	CIN3	SCC
31-40	05	04	00	00
41-50	03	02	03	02
51-60	01	00	02	02
61-70	00	00	00	01
TOTAL	09	06	05	05

CIN-1 shows cervical squamous mucosa with koilocytes in the superficial upper layers (Fig 1(A)). Squamous mucosa with koilocytic atypia in the superficial layer, underlying endocervical gland is normal. (Fig 1 (B)).

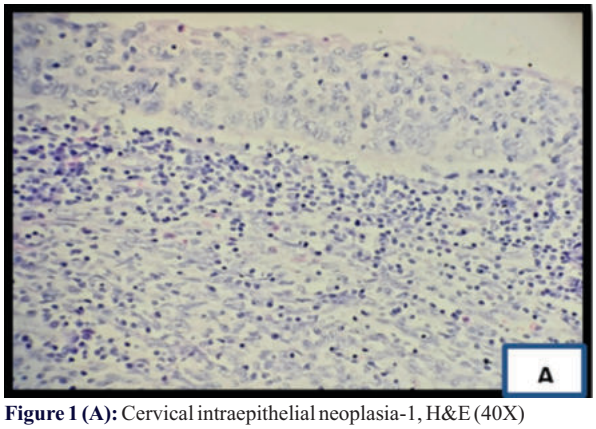

Figure 1 (A): Cervical intraepithelial neoplasia-1, H&E (40X)

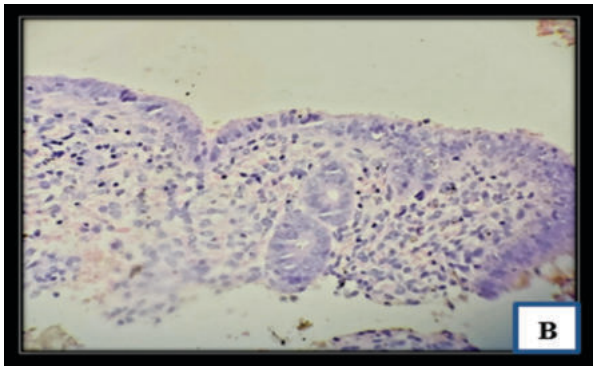

Figure 1(B): Cervical intraepithelial neoplasia-1, H&E (4OX).

CIN-2 shows dysplastic cellular changes in lower half or lower two-thirds of epithelium with marked nuclear abnormalities (Fig 2).

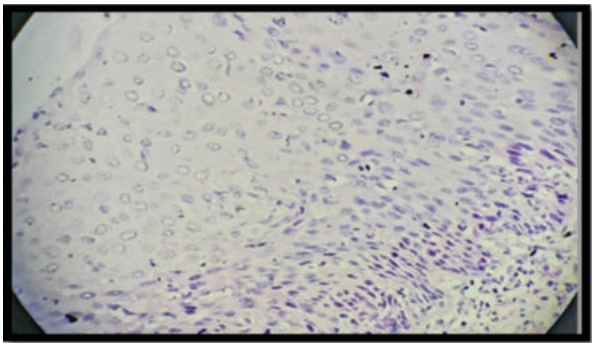

Figure 2: Cervical intraepithelial neoplasia-2, H&E (40X)

CIN-3 shows differentiation and stratification absent in superficial epithelium with mitotic figures. Nuclear abnormalities extend throughout epithelium (Fig 3 (A), (B).

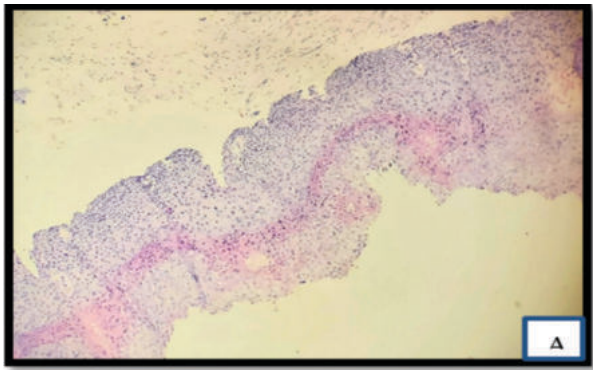

Figure 3(A): Cervical intraepithelial neoplasia-3, H&E (10X).

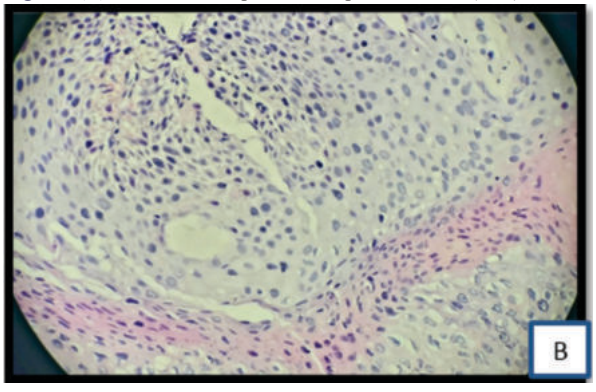

Figure 3(B): Cervical intraepithelial neoplasia-3, H&E (40X).

Squamous cell carcinoma cervix shows invasive epithelial tumor composed of neoplastic cells with varying degrees of squamous differentiation. (Fig 4 (A), (B)).

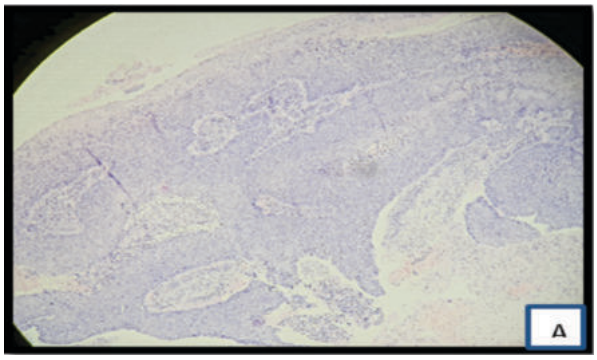

Figure 4 (A): Squamous cell carcinoma cervix, H&E (10X).

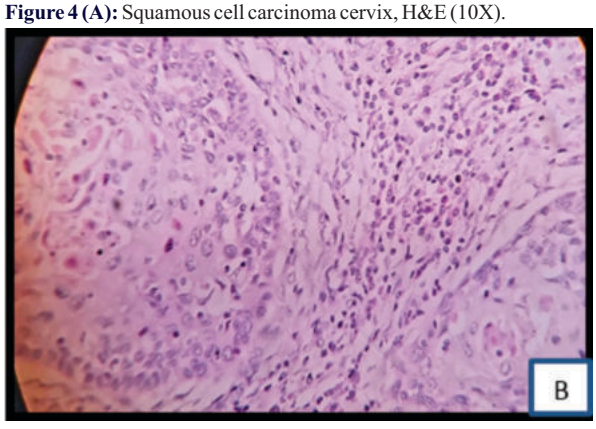

Figure 4 (B): Squamous cell carcinoma cervix, H&E (40X).

In our study among 25 cases taken for IHC staining using p16 antibody which were CIN 1 , CIN 2 , CIN 3 , Squamous cell carcinoma , total of 18 cases were positive Among positive cases of CIN as level of dysplasia increases there is increase in intensity of immunostaining. In CIN1 among 9 cases, 5 cases were positive (55.5% positivity). In CIN2 among 6 cases, 4 cases were positive (66.6% positivity). In CIN3 among 5 cases, 4 cases were positive (80% positivity). In SCC all 5 cases were positive (100% positivity) (Table3).

Table 3: P16 evaluation among different groups			
HPE Diagnosis	No. of Cases	P16 Positivity	Percentage
CIN 1	09	05	55.5%
CIN 2	06	04	66.6%
CIN 3	05	04	80%
SCC	05	05	100%

Intensity Of Staining Among Positive Cases –
CIN1 cases show predominantly weak intensity. CIN 2 moderate intensity whereas CIN 3 and SCC show predominantly strong intensity. A progressive increase of P16 expression is seen with grade of CIN (Table 4). P16 correlates with grade of CIN and is a sensitive marker of Cervical Intraepithelial Neoplasia.

Table 4 : Intensity of staining			
HPE diagnosis	1+	2+	3+
CIN 1	04 (80%)	01(20%)	0
CIN 2	01 (25%)	02 (50%)	01 (25%)
CIN 3	0	01 (25%)	03 (75%)
SCC	0	0	05(100%)

Pattern Of Staining –
CIN 1 show predominantly patchy positivity, CIN 2 show predominantly diffuse basal positivity and CIN3, SCC predominantly show diffuse full thickness positivity.

As the level of dysplasia increases there is increase in intensity of immunostaining (Table 5).

Table 5 : Pattern of staining			
HPE diagnosis	Patchy	Diffuse basal	Diffuse full thickness
CIN 1	04	01	0
CIN 2	01	02	01
CIN 3	0	01	03
SCC	0	0	05

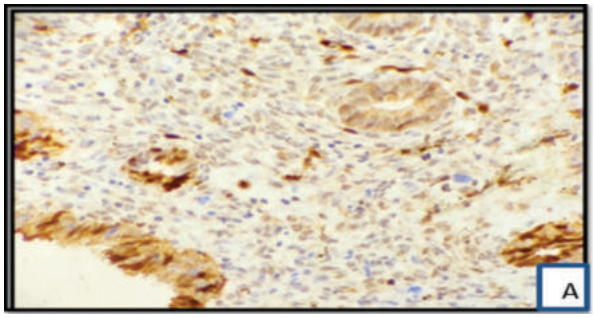

Fig 5 (A): Cervical intraepithelial neoplasia – 1, IHC P16 (40X) - Patchy positivity

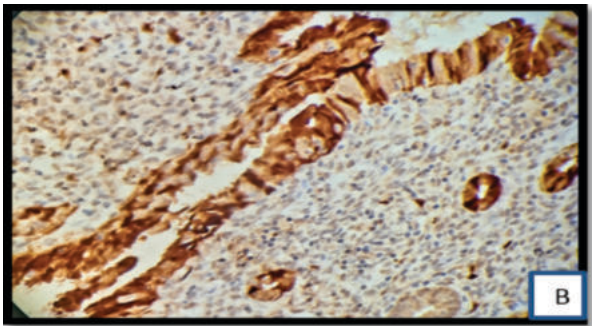

Fig 5 (B): Cervical intraepithelial neoplasia – 1, IHC P16 (40X) - Diffuse basal positivity

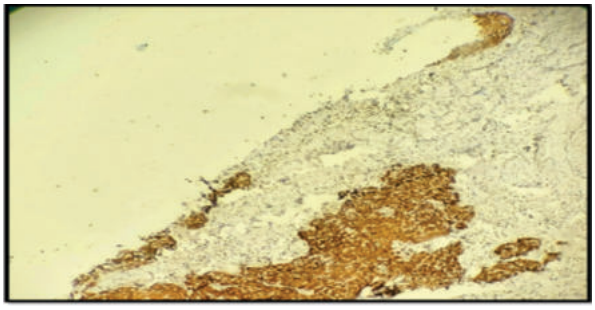

Fig 6: Cervical intraepithelial neoplasia – 2, IHC P16 (40X)-Diffuse basal positivity

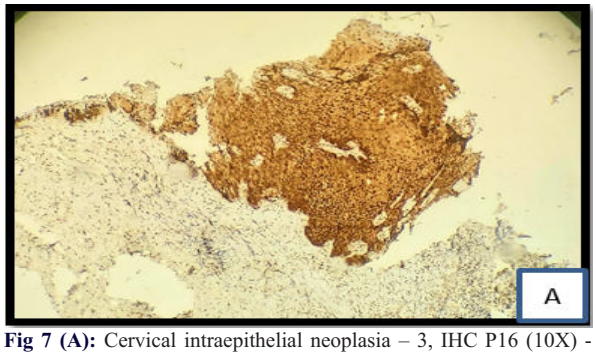

Fig 7 (A): Cervical intraepithelial neoplasia – 3, IHC P16 (10X) - Diffuse full thickness positivity

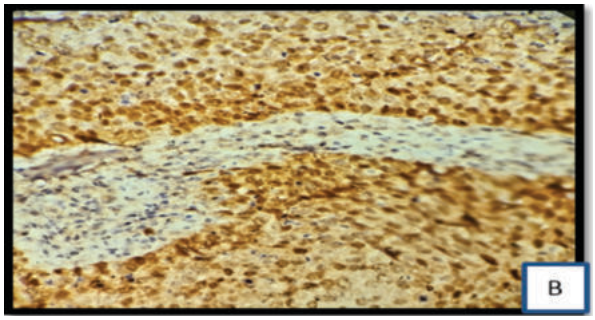

Fig 7(B): Cervical intraepithelial neoplasia – 3, IHC P16 (40X)- Diffuse full thickness positivity, Nuclear cytoplasmic positivity.

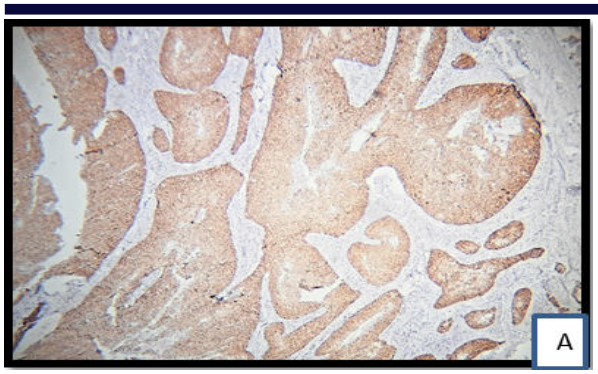

Fig 8 (A) Squamous cell carcinoma, IHC P16 (10X) - Diffuse full thickness positivity

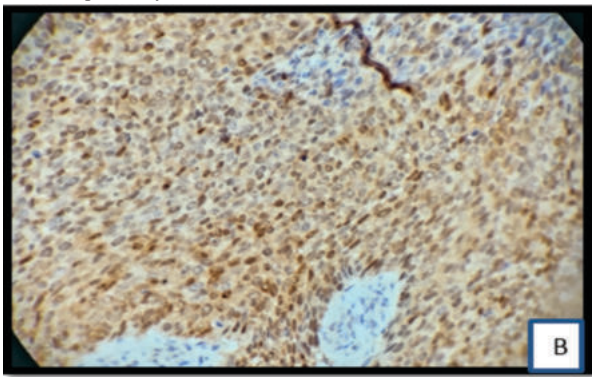

Fig 8 (B) Squamous cell carcinoma, IHC P16 (40X)-Diffuse full thickness positivity, Nuclear cytoplasmic positivity.

DISCUSSION:

Several studies have reported p16 overexpression in cervical cancer as well as in high grade cervical intraepithelial neoplasia (CIN2-3). P16 can be useful for identifying high grade lesion when histological features are equivocal. The strong association between the degree of dysplasia and p16 immunoreactivity in the CIN specimens could be explained by cervical malignant transformation associated with high-risk HPV infections due to high levels of the E7 viral oncogene. Cervical p16 staining with high-risk HPV types has been reported as strong, diffuse and located either in nuclei and/or cytoplasm compartments. Differently, in infections caused by low-risk HPV types, the p16 immunostaining pattern displayed weak and focal staining, located in nuclei and cytoplasm of the intermediate and superficial layers only.[6]

In a study by Klaes et al. demonstrated that p16 identifies dysplastic cervical epithelia. [7] In another study, the same group reported that p16 immunostaining allows for a precise identification of small CIN or cervical cancer lesions. [8] Amaro-Filho et al. observed p16 overexpression in invasive cervical cancer, with diffuse patterns and strong intensity, when compared to the control samples. Another study in CIN-1 showed that specimens with diffuse p16 immunostaining had higher chances of progression to CIN2-3. [9]

Mills et al recommend that p16 should only be used selectively for problematic scenarios, such as CIN 2 because of its inherent lack of reproducibility, especially in cases in which CIN 1 and CIN 2 are benign mimics of CIN3.[10] Liu et al stated that p16 can be correlated with clinicopathological and prognosis of cervical cancer.[11]

In our study using IHC analysis, we evaluated the expression of p16INK4a in samples of cervical biopsies from 25 patients with CIN 1, CIN 2, CIN 3 and SCC. Our findings showed that the expression of p16 increases from normal to squamous carcinoma in the uterine cervix emphasizing that it might be a useful marker for predicting risk of developing cervical cancer in women. All the cases of SCC in this study expressed p16. (Table 6).

IHC has had a major impact in the field of uterine cervical pathology. IHC should always be used as an adjunct to morphological examination and the results should not be interpreted in isolation. Of great interest for routine diagnostic use is the fact that IHC testing for p16 to identify positive cells has the potential to recognize those lesions with increased risk of progression to high-grade lesions.

Table 6: Studies related to p16 expression

References	Histology	Conclusions
KLAES et al,(2001)[7]	Inflammation/CIN1/CIN2/ CIN3/SCC	P16 positively correlated with grade of cervical lesion P16was more useful for CIN 2-3
KLAES et al, (2002)[8]		
AMARO-FILHO et al, (2013)[9]	Inflammation/CIN1/CIN2/ CIN3/SCC/Other tumors	P16 was more expressed in advanced FIGO stages
MILLS et al, (2015)[10]	CIN 1,2,3	P16 should be used for CIN2-3
LIU et al , (2015)[11]	Normal/CIN1/CIN2/CIN3 /SCC	P16 positively correlated with the grade of cervical diseases.
Present study 25 cases	CIN1/CIN2/CIN3/ SCC	P16 positively correlated with the grade of cervical lesion

CONCLUSION:

P16 serve as a biomarker that is independent of individual HPV type and indicative of cervical cancer disease process in action.

There are many types of HPV but the effect of E7 oncoprotein is same in blocking pRB and leading to overexpression of P16.P16 immunostaining allowed precise identification of even small CIN in biopsy sections and reduces interobserver variation in histopathologic interpretation.

P16 predicts the risk of those progressing to carcinoma. It is important as only high grade lesions require further intervention and treatment while negative cases may be followed up with screening.

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