

## ASSOCIATION OF HUMAN PAPILLOMA VIRUS INFECTION IN UTERINE CERVICAL NEOPLASIA- A CROSS SECTIONAL STUDY

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### ABSTRACT

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#### BACKGROUND

Cervical cancer is the second most frequent cancer in women in the world. The association of Human Papilloma Virus (HPV) infection with cervical carcinogenesis is well documented and nearly all cervical cancers are caused by persistent infection with some high-risk HPV types. In developed countries, there has been a major decline in cervical-cancer mortality after the introduction of large-scale cytological testing but in developing countries, due to lack of effective screening programmes for cervical cancer no clinically significant reduction in the incidence of cervical cancer has occurred during the past three decades. Pap smear is a routine screening test, but the overall sensitivity in detection of HSIL - precancerous lesion is only 70 to 80% and histopathological diagnosis of cervical biopsy is prone to poor inter observer reproducibility. Immunohistochemical staining for biomarkers like p16INK4a a sensitive marker for Human Papilloma Virus, may provide objective standards to reduce diagnostic variability of cervical biopsy evaluations.

Objectives of the study were to study the proportion of HPV infection in uterine cervical neoplasia using immunohistochemistry for the marker p16INK4a in cervical biopsy specimens and to assess the proportion of P16 expression in varying grades of cervical neoplasia included in the study.

#### MATERIALS AND METHODS

The study was conducted in the department of pathology, Govt. Medical College, Kottayam for a duration of one year. Study sample included 50 cases of uterine cervical biopsy specimens, histopathologically diagnosed as cervical epithelial neoplasia, received in the Department of Immunohistochemistry for the marker p16INK4a. Immunohistochemistry for the marker p16INK4a was done as per Standard Operating Procedures in all 50 cases and the expression of this protein which is a sensitive marker for Human Papilloma Virus, was studied. 50 cases in which the p16 expression was studied were categorized into p16 positive and negative cases and their histomorphological parameters were compared. The factors studied were age, histological type, differentiation status of tumour, P16 expression and its immunohistochemical score. Statistical analysis is done using SPSS version 17.

#### RESULTS

Summarizing the study results, 87% of Squamous Cell Carcinoma studied came under >50 years group. Whereas <50 years group included 70% of LSIL and 83.3% of Squamous metaplasia. Well differentiated SCC, moderately differentiated SCC and poorly differentiated SCC were 40%, 57% & 3% respectively. Out of the 50 cases in which p16 IHC study was done, 70% were positive and 30% were negative, from which the prevalence of HPV in our study population is inferred as 70%.

In our study 90% cases of SCC and 50% of LSIL were p16 positive. Single case studied each of HSIL, Basaloid SCC and Adenocarcinoma cervix showed positivity for p16. Squamous metaplasia which is the benign epithelial lesion studied showed 100% p16 negativity. 50% of LSIL and the single adenosquamous carcinoma studied were p16 negative. All these results are comparable with other studies. In present study, 3/27 cases of SCC were negative for p16 which is also comparable with other Indian studies.

Increasing P16 score was noticed as moving from benign to malignant through premalignant neoplasms. In present study, P16 expression among various degree of differentiation in Squamous Cell Carcinoma did not show statistically significant association, but indicated a trend of increased frequency of positive p16 expression as moving from well differentiated to moderate/poorly differentiated SCC, which is statistically significant.

#### CONCLUSION

Present study confirms the association of Human Papilloma Virus (HPV) infection and cervical malignant and premalignant neoplasia which is well recognized worldwide. It also recognizes P16INK4a as a sensitive marker for the presence of HPV and its expression was observed in squamous cell carcinoma, HSIL, LSIL and adenocarcinoma cervix. In this study, increased p16 expression (P16 IHC score) was observed in cervical epithelial neoplasia with increasing severity of lesion. Also, no positive staining of p16 was observed in metaplastic cervical epithelium and so may be helpful in distinguishing from squamous intra epithelial lesions. To conclude, combined use of H&E and P16 immunohistochemistry significantly improves the accuracy of interpreting and grading cervical lesion biopsies and may be incorporated into routine diagnostic/screening algorithm for cervical carcinoma.

**KEYWORDS**

Cervical Biopsy, Immunohistochemistry, Human Papilloma Virus, P16INK4a, Cervical Epithelial Neoplasia.

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**BACKGROUND**

Cervical cancer is the second most frequent cancer in women in the world with approximately 0.5 million cases worldwide, and it is the third largest cause of death from cancer in women.<sup>1</sup> Average age of the patients with invasive cervical carcinoma is 45 years.

The association of Human Papilloma Virus (HPV) infection with cervical carcinogenesis is well documented.<sup>2</sup> Among 200 different subtypes of HPV, nearly all cervical cancers are caused by persistent infection with some high-risk HPV types.<sup>3</sup> Squamous Cell Carcinoma is the most common histological sub type accounting for approximately 80%. Although most HPV infections clear up on their own and most pre-cancerous lesions resolve spontaneously, there is a risk for all women that HPV infection may become chronic and pre-cancerous lesions progress to invasive cervical cancer. It takes 15 to 20 years for cervical cancer to develop in women with normal immune systems. This slow progression gives ample time for screening, detection and preventive treatment.

In developing countries, there is a lack of effective screening programmes for cervical cancer and so no clinically significant reduction in the incidence of cervical cancer has occurred during the past three decades.<sup>4</sup> In developed countries, by contrast, there has been a major decline in cervical-cancer mortality after the introduction of large-scale cytological testing.<sup>5</sup> There are currently two vaccines which protect against both HPV 16 and 18, which are known to cause 70% of cervical cancers. WHO recommends that in countries where HPV vaccine is introduced, screening programmes may still need to be developed or strengthened.

Pap smear is a routine screening test, but the overall sensitivity in detection of HSIL - precancerous lesion is only 70 to 80%.<sup>6</sup> Histopathological diagnosis of cervical biopsies determines clinical management of patients with an abnormal cervical cancer-screening test and it is prone to poor inter observer reproducibility. High cost and restricted use of standardized diagnostic test for HPV also contributes to the cancer deaths. Immune histochemical staining for biomarkers related to the different stages of cervical

carcinogenesis may provide objective standards to reduce diagnostic variability of cervical biopsy evaluations.<sup>7</sup>

P16INK4A, a CDK inhibitor protein is a biomarker of HPV E7 oncoprotein activity. Over expression of P16INK4A (from now will be called as p16) is observed when pRb is inactivated by E7 oncoprotein of oncogenic HPV types. P16 is the surrogate marker for the oncogenic process for HPV replication competent cells of cervical epithelium and it's over expression was well established in CIN and in invasive SCC.<sup>8</sup> The immunohistochemical identification of high-risk (HR)HPV by a surrogate marker like p16INK4a could be a more affordable alternative to HPV-DNA testing.

**Aims and Objectives****Primary Objective**

To study the proportion of HPV infection in uterine cervical neoplasia detected by immunohistochemistry in 50 biopsy specimens received in histopathology department of Government Medical College Kottayam, during one year.

**Secondary Objective**

To assess the proportion of HPV infection by immunohistochemistry in varying grades of uterine cervical neoplasia included in the study.

**MATERIALS AND METHODS****Study Period**

A study period of one year from August 2014 to September 2015.

**Sample Size**

50 cases of uterine cervical biopsy specimens histopathologically diagnosed as cervical epithelial neoplasia.

**Settings**

The Department of Pathology of Government Medical College, Kottayam.

**Study Tool**

Uterine cervical biopsy specimens histopathologically diagnosed as cervical epithelial neoplasia received in the Department of Pathology of Government Medical College Kottayam during one-year period, the cervical biopsies were done in the department of Gynaecology of the same institution.

**Methodology**

Study sample include 50 cases of Uterine cervical biopsy specimens histopathologically diagnosed as cervical epithelial neoplasia received in the Department of Pathology of Government Medical College, Kottayam during one-year

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period, The cervical biopsies were done in the department of Gynaecology of the same institution. All specimens are received in formalin, processed and paraffin embedded in total. Thin 3-4 micrometer sections are taken and stained by Haematoxylin and Eosin. Immunohistochemistry for the marker p16INK4a (mouse monoclonal P16 PM 143FB15A) was done in all 50 cases and the expression of this protein which is a sensitive marker for Human Papilloma Virus was studied. Using mouse monoclonal antibody to p16, microwave aided antigen retrieval and standard immune peroxidase technique, formalin fixed paraffin embedded tissue sections are immune-stained as per Standard Operating Procedures.

Variable degree of p16 staining was seen in the cervical stromal fibroblasts which served as an internal control and cervical biopsy with no epithelial lesion was used as negative control in the initial run.<sup>8</sup> In subsequent runs, cases that were found to be positive were also kept as positive controls. The clinical details of the 50 cases were collected by reviewing the medical records. After examining the haematoxylin and eosin stained slides the tumours were categorized into different groups. Malignant tumours were also classified as well differentiated, moderately differentiated or poorly differentiated depending upon the morphology. The 50 cases in which the p16 expression is studied were categorized into p16 positive and negative cases and their histomorphological parameters were compared. The factors studied were age, histological type, differentiation status of tumour, P16 expression and its immunohistochemical score.

The proportion of tumours showing p16 positivity is expressed as percentage. Statistical analysis is done using SPSS version 17. Chi- square test is used to find if there is any statistically significant association between p16 expression and the parameters studied.

**Interpretation of P16 IHC Staining Results<sup>8</sup>**

Result is interpreted by examining the development of brown colour in tissue sections, its intensity, positive tumour cell percentage and location.

Degree of nuclear staining and/or cytoplasmic staining for the marker is considered.

**Scoring was done as follows:**

Negative: No staining

Positive:

1+ - 1-5% of cells stained

2+ - 5-25% of cells stained

3+ - >25% of cells stained

Intensity of immunostaining was taken as 1+, 2+ and 3+ depending upon the positivity.

Immunohistochemistry score is calculated as a product of percentage positive tumor cells (0-3) and staining intensity score (0-3) thus achieving a maximum of 9.

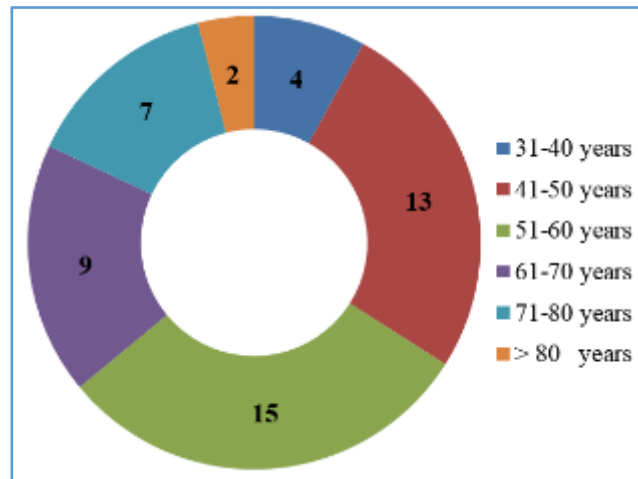
**Inclusion Criteria**

1. Biopsy specimens histopathologically diagnosed as uterine cervical epithelial carcinoma.
2. Biopsy specimens histopathologically diagnosed as uterine cervical epithelial neoplasm.

**Exclusion Criteria**

Uterine cervical lesions other than epithelial neoplasms.

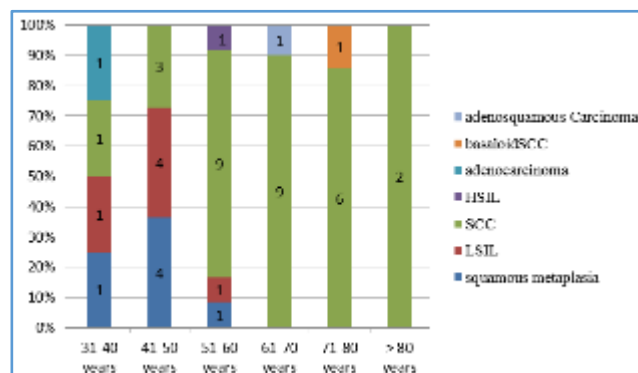
**RESULTS**



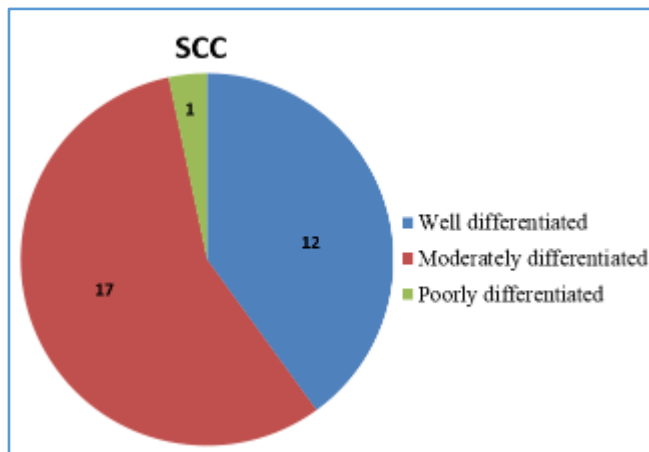
**Graph 1. Age Distribution of Patients with Cervical Epithelial Neoplasia**

Type of Epithelial Neoplasia	Frequency	Percent
Squamous metaplasia	6	12%
LSIL	10	20%
HSIL	1	2%
Squamous cell carcinoma	30	60%
Adeno carcinoma	1	2%
Basaloid SCC	1	2%
Adeno squamous carcinoma	1	2%
Total	50	100%

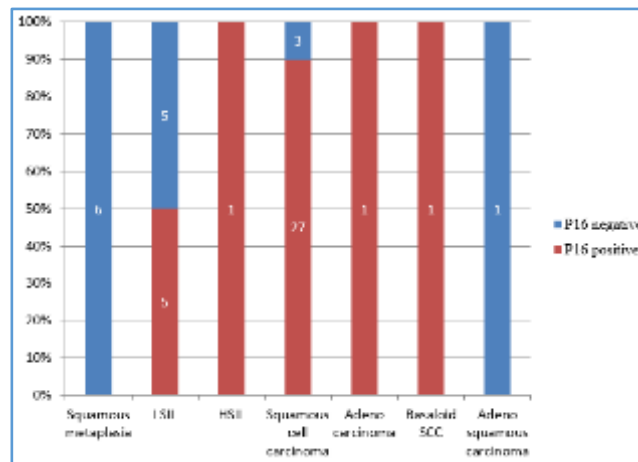
**Table 1. Distribution of Histologic Type of Cervical Epithelial Neoplasia**



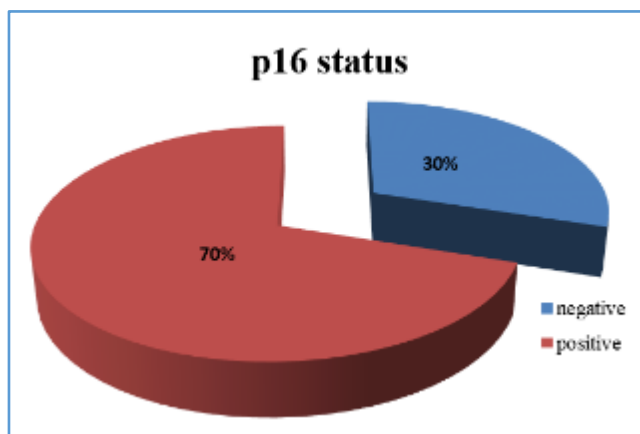
**Graph 2. Percentage Distribution of Different Histologic Type of Cervical Epithelial Neoplasia in Various Age Groups**



**Graph 3. Frequency Distribution of Differentiation Status in Squamous Cell Carcinoma**



**Graph 5. P16 Expression Among Histological types of Cervical Epithelial Neoplasia**

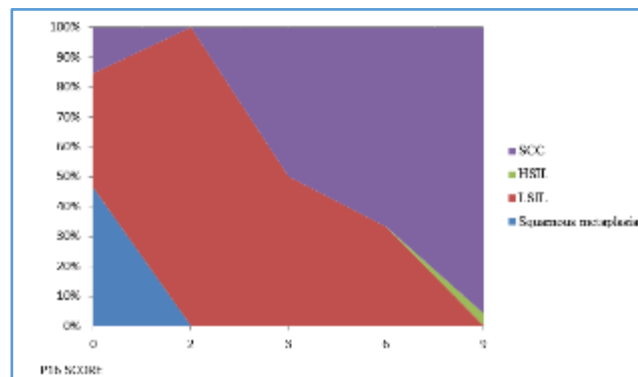


**Graph 4. P16 Status of the Cases in Which the Marker was Studied**

P16 Score	0	2	3	6	9
Squamous metaplasia	6	0	0	0	0
LSIL	5	1	3	1	0
HSIL	0	0	0	0	1
Squamous Cell Carcinoma	3	0	2	2	23

**Table 3. P16 Immunohistochemical Score in Squamous Epithelial Neoplasia**

Chi-square value= 54.08 with p value= 0.001.  
 Linear-by-Linear Association= 18.10 with p value= 0.001.



**Graph 6. Trend of P16 Immunohistochemical Score in Squamous Epithelial Neoplasia**

Type of Epithelial Neoplasia	P16 status		Total
	Positive	Negative	
Squamous metaplasia	0	6 (100%)	6
LSIL	5 (50%)	5 (50%)	10
HSIL	1(100%)	0	1
Squamous Cell Carcinoma	27 (90%)	3 (10%)	30
Adeno carcinoma	1(100%)	0	1
Basaloid SCC	1(100%)	0	1
Adeno squamous carcinoma	0	1(100%)	1

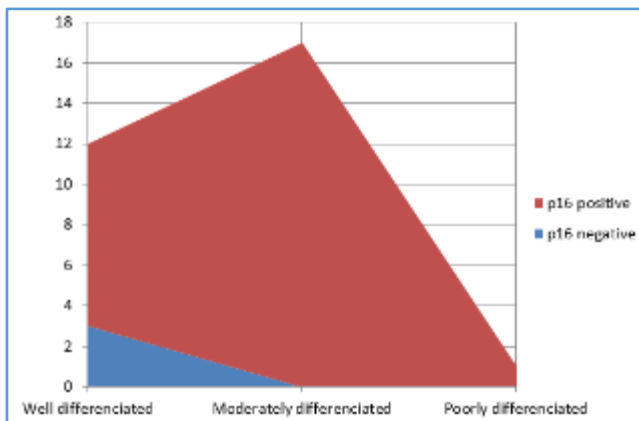
**Table 2. Association between P16 Status and Different Histologic Type of Cervical Epithelial Neoplasia**

Chi-square value= 25.238 p value= 0.001.

p16 Status	Well Differentiated	Moderately Differentiated	Poorly Differentiated
Negative	3	0	0
Positive	9	17	1

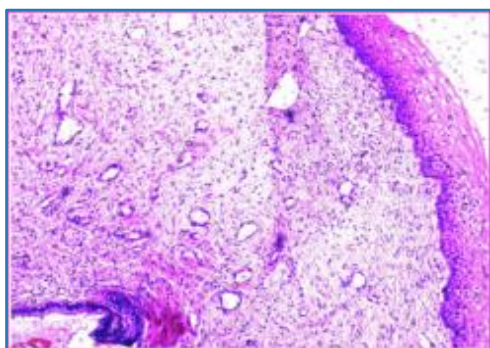
**Table 4. P16 Expression Among Various Degree of Differentiation in Squamous Cell Carcinoma**

Chi-square value= 5.00 with p value= 0.082.  
 Linear-by-Linear Association= 4.324 with p value= 0.038.

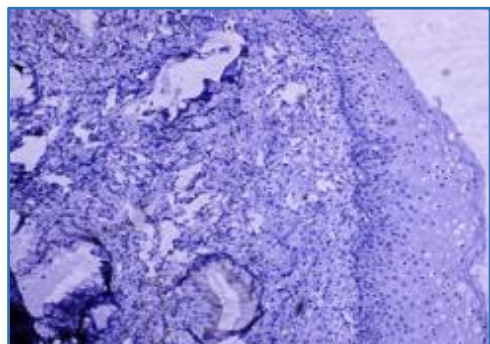


**Graph 7. Trend of P16 Expression in Various Degree of Differentiation of Squamous Cell Carcinoma**

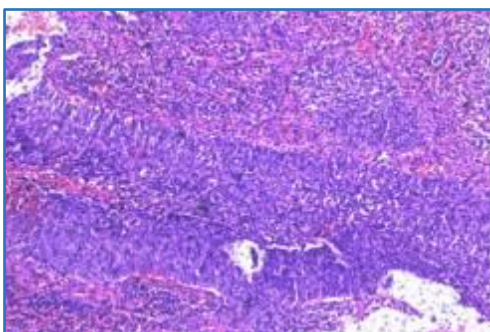
**Photomicrographs-**



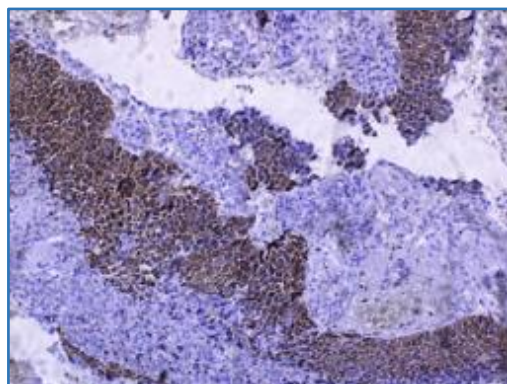
**Figure 1. 50 year, Squamous Metaplasia, Cervix, H & E: 20X**



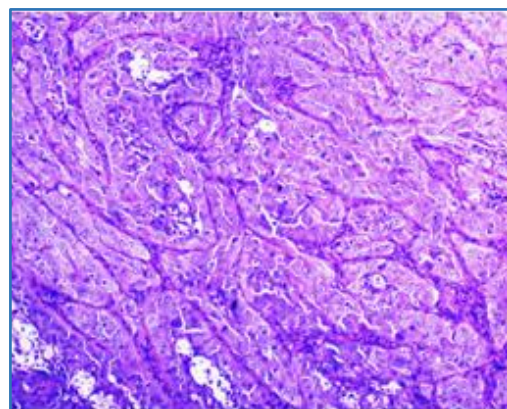
**Figure 2. IHC of the Case in Figure 1- Negative for p16:20X**



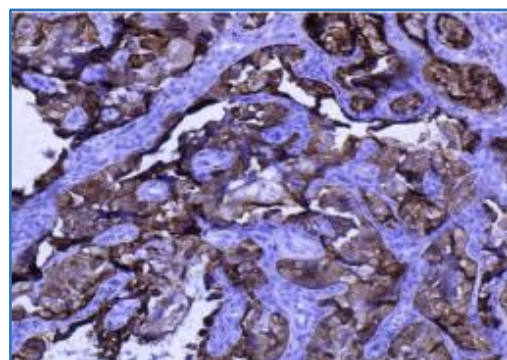
**Figure 3. 55 years, Moderately Differentiated Squamous Cell Carcinoma, Cervix; H&E: 20X**



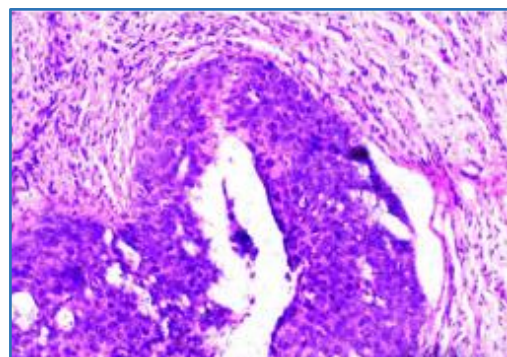
**Figure 4. IHC of the Case in Figure 3- Negative for p16:20X**



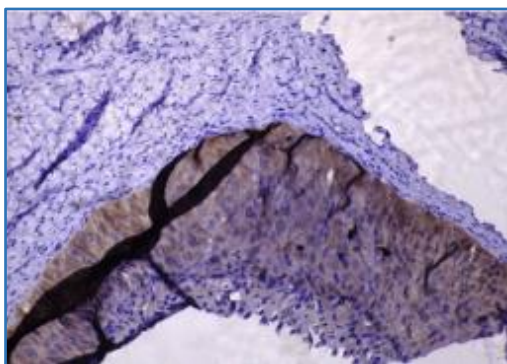
**Figure 5. 75 year, Poorly Differentiated Squamous Cell Carcinoma, Cervix; H&E: 20x**



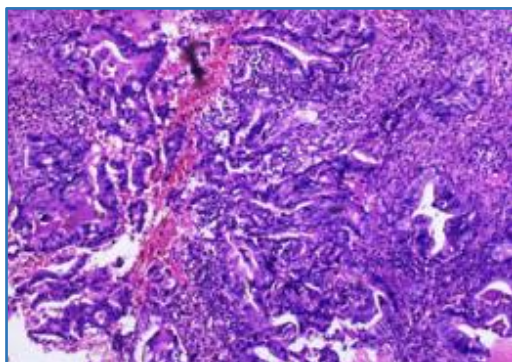
**Figure 6. IHC of the Case in Figure 5- Negative for p16:20X**



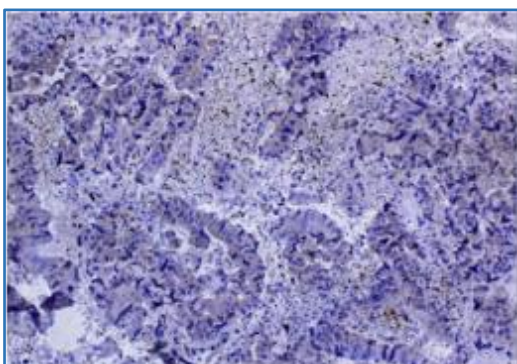
**Figure 7. 54 year, HSIL Cervix; H&E: 20x**



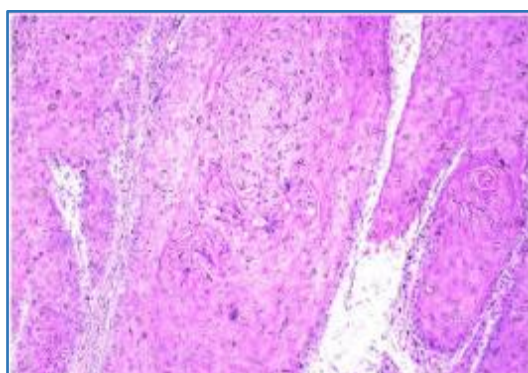
**Figure 8. IHC of the Case in Figure 7- Negative for p16:20X**



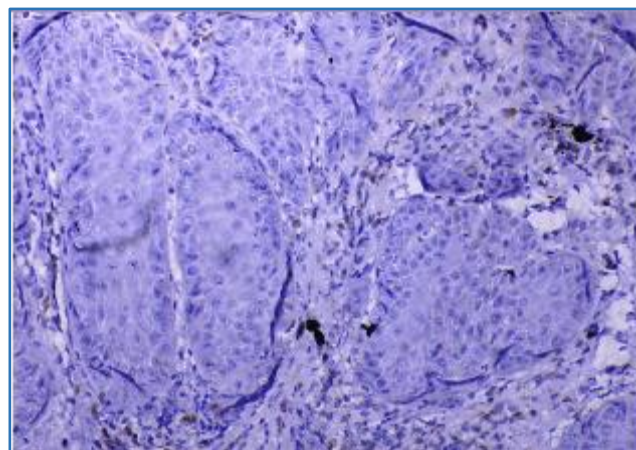
**Figure 9. 39 Year, Well Differentiated Adeno Carcinoma, Cervix; H&E: 20x**



**Figure 10. IHC of the Case in Figure 9- Negative for p16:20X**



**Figure 11. 85 year, Well Differentiated Squamous Cell Carcinoma, Cervix; H&E: 20x**



**Figure 12. IHC of the Case in Figure 11- Negative for p16:20X**

**DISCUSSION**

In this study, 50 cases of uterine cervical neoplasia were considered. Their histomorphological features were studied. In all cases p16INK4a IHC was done and the expression of this protein which is a sensitive marker for Human Papilloma Virus was studied.

They were categorized into p16 positive and negative cases and their histomorphological parameters and degree of P16 expression were compared.

**Age Distribution**

The percentage distribution of different histological type of cervical epithelial neoplasia in various age groups and highlights that the precancerous lesions clustered in 40-60 years. These are comparable with earlier Indian studies.<sup>9</sup>

**Tumour Histology**

In the Present Study, Squamous Cell Carcinoma constituted 60% of cervical epithelial neoplasia. LSIL and Squamous metaplasia contributed 20% and 12% respectively and 2% each contributed by HSIL, Basaloid SCC, Adenosquamous carcinoma and Adenocarcinoma cervix. 40% of the SCC were well differentiated, 57% moderately differentiated and 3% were poorly differentiated.

**P16 Expression and HPV Prevalence**

Out of the 50 cases in which p16 IHC study was done, 70% were positive and 30% were negative, from which the prevalence of HPV in our study population is inferred as 70%. Most of the cases showed 2+ to 3+ positivity for the marker. This is comparable to 75-100% expression reported in most of the earlier studies.<sup>10</sup>

**Association between p16 Positivity and Tumour Histology**

In current study 90% cases of SCC and 50% of LSIL were p16 positive. Single case studied each of HSIL, Basaloid SCC and Adenocarcinoma cervix showed positivity for p16. Squamous metaplasia which is the benign epithelial lesion studied showed 100% p16 negativity. 50% of LSIL and the single adenosquamous carcinoma studied were p16 negative. These observations are found to be statistically significant. Present study findings are similar to other studies.<sup>11,12</sup>

	Normal Positivity	CIN1 Positivity	CIN2 Positivity	CIN3 Positivity	SCC Positivity
Focchi et al, 2007	12%, 7/58	91%, 80/88	100%, 65/65	ND	100%, 47/47
Benevolo et al, 2006	ND	31%, 17/54	90%, 9/10	ND	100%, 11/11
Tringler et al, 2004	6.5%, 7/108	72%, 13/18	100%, 46/46	ND	100%, 18/18
Murphy et al, 2005	ND	100%, 38/38	100%, 33/33	98%, 45/46	100%, 10/10
Agoff et al, 2003	11%, 19/133	57%, 43/76	75%, 60/80	91%, 103/113	92%, 42/46
Klaes et al, 2001	ND	81% 1/ 47	0% 0/32	100% 60/60	98% 52/53
Present Study	0%, 0/6	50%, 5/10	ND	100%, 1/1	90%, 27/30

**Table 5. Summary of Recent Studies on P16 Expression in Various Cervical Epithelial Lesions and Comparison of the Present Study**

ND – Not done.

In the present study, 3/27 cases of SCC were negative for p16 in which technical reasons for negativity have been excluded as these cases showed positive internal control. In a recent study by Kang et al 35.7 % and 28% of CIN 2 and CIN 3 cases were negative for p16 expression. The lack of immunoreactivity was correlated to promote hypermethylation. In their study they concluded that the p16 silencing during CIN was not a rare event and also does not correspond with either HPV status or CIN grading.

#### Association between p16 score and Types of Tumour

P16 immunohistochemical score (0-9) in various squamous epithelial neoplasia which were positive for P16 showed a Linear-by-Linear Association of 18.10 with p value of 0.001 indicating an increasing P16 score as moving from benign to malignant through premalignant neoplasms, which is statistically significant. Comparable results were also found in other studies.<sup>8,13</sup>

#### Association between p16 Positivity and Tumour Differentiation in SCC

P16 expression among various degree of differentiation in squamous cell carcinoma did not show statistically significant association with a p value of 0.082, but showed a Linear-by-Linear Association of 4.324 with p value of 0.038 indicating a trend of increased frequency of positive p16 expression as moving from well differentiated to moderate/poorly differentiated SCC, which is statistically significant.

#### CONCLUSION

- The association of Human Papilloma Virus (HPV) infection and cervical malignant and premalignant neoplasia is well recognized worldwide and present study confirms this.
- P16 status assessment may be incorporated into routine diagnostic/screening algorithm for cervical carcinoma for the following reasons
  - P16INK4a is a sensitive marker for the presence of HPV and its expression was observed in squamous cell carcinoma, HSIL, LSIL and adenocarcinoma cervix.
  - Increased p16 expression (P16 IHC score) was observed in cervical epithelial neoplasia with increasing severity of lesion.
  - No positive staining of p16 was observed in metaplastic cervical epithelium and so may be helpful

in distinguishing from squamous intra epithelial lesions.

- Combined use of H&E and P16 immunohistochemistry significantly improves the accuracy of interpreting and grading cervical lesions on biopsies and can be carried out in clinical settings where molecular methods for viral DNA detection are unavailable.

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