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A STUDY OF p16 EXPRESSION IN PREMALIGNANT AND MALIGNANT LESIONS OF UTERINE CERVIX

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ABSTRACT

Cervical cancer is the fourth most common cancer in women worldwide and a leading cause of cancer-related deaths in developing countries. Persistent infection of Human Papilloma Virus (HPV) is a significant casual agent for cervical cancer development. High-risk HPV types are associated with precancerous cervical lesions and invasive cervical cancers, while low-risk HPV types are associated with low-risk cervical lesions. The most effective preventive strategy for cervical cancer is systematic screening for women aged 30-59. This study aims to study the association of cervical malignancies and HPV infection using p16 immunostaining and evaluate its etiological and prognostic benefits. From January 2017 to June 2018, 54 cervical biopsy/hysterectomy specimens were submitted to MIMS for histopathological evaluation. Clinical data was obtained, and gross features were assessed. The tissue was paraffin embedded, stained with H and E stain, and studied according to the proforma. Discussion: The study found that the mean age of patients with carcinoma cervix was 52-11 years, with the peak incidence occurring in the 5th decade. Most patients were of parity 4-5, consistent with previous studies. The most common clinical presentation was WDPV and intermenstrual bleeding in pre-menopausal women, while PMB and WDPV were the most common in postmenopausal women. The majority of cases were squamous cell carcinoma (85.2%), followed by CIN III (3.7%), adenocarcinome (1.9%), adenosquamous cell carcinoma (1.9%), and CIN I (1.9%).

The study assessed p16 expression in 54 cases of carcinoma cervix and found a significant correlation between p16 expression subtypes and invasive carcinoma. The p16 positivity increased with age and parity, but not statistically significant. P16 expression was found in premalignant and malignant cervical lesions, with greater and stronger expression in invasive cervical carcinoma and squamous cell carcinomas. Further studies with large samples and correlating p16 expression with clinicopathological parameters are needed to develop targeted therapy for carcinoma cervix.

Keywords : Human papilloma virus; Immunohistochemistry ; p16; cervical carcinoma; clincopathological prognostic parameters**.**

INTRODUCTION:

Cervical cancer is the fourth most common cancer in women worldwide and is one of the leading causes of cancer related death of women in developing countries[1]. In India, the incidence of cervical cancer significantly rises around the age of 45 and peaks at 55 years of age[2]. Persistent infection of Human Papilloma Virus (HPV) is considered the most significant and 'necessary' casual agent for the development of cancer of uterine cervix. 15 HPV types classified as 'high-risk' types are associated with precancerous cervical lesions and invasive cervical cancers, while 6-11 HPV types are associated with low-risk cervical lesions[3]. HPV types 16 and 18 are the two most common oncogenic HPV types found in invasive cervical cancer and high-grade cervical intraepithelial neoplastic (CIN) lesions.[2][7]

ISSN: 0975-3583,0976-2833 VOL14, ISSUE 08, 2023

The most effective preventive strategy for cervical cancer is systematic screening of women between 30 and 59 years of age. There are various biomarkers of cervical carcinoma, including HPV DNA and secondary markers like p53, c-fos, p50, fra 1, p16, notch 1, rb and telomerase[4]. The p16 protein is a tumor suppressor which negatively controls cell cycle progression at the G1/S checkpoint[5]. This study aims to study the association of cervical malignancies and HPV infection by using p16 immunostaining and evaluate its etiological and prognostic benefits as a valuable marker for cervical carcinoma[6]. The study also aims to correlation of immune positivity of p16 with the histo-pathological type, grade of carcinoma cervix and clinico-pathological prognostic parameters.

AIMS AND OBJECTIVES:

1. To Study the pattern of expression of p16 in premalignant and malignant lesions of cervix by immunohistochemical staining.
2. To determine the percentage of p16 expression in various histologic types of cervical carcinomas.
3. Correlation of p16 expression with the histopathological grades of cervical carcinomas.

MATERIALS AND METHODS:

54 cervical biopsy/hysterectomy specimens were submitted to the Department of Pathology, MIMS, Mandya for histopathological evaluation from January 2017 to June 2018. Clinical data was obtained from patient records and requisition forms. The specimens were fixed in 10% neutral buffer formalin and the evaluation of gross features was done. The gross details of specimens submitted for evaluation of malignancy were observed and recorded based on the protocol for evaluation of cervical malignancy.

The tissue from hysterectomy and cervical biopsy specimens was paraffin embedded, stained with H and E stain and studied according to the proforma.

Procedure for H and E staining

- Deparaffinise in Xylene – 2 changes – 5 minutes each.
- Wash in absolute alcohol – 1 change – 3 minutes.
- Wash in water for 3-5 minutes.
- Stain with haematoxylin for 5 minutes.
- Place in running tap water for bluing for 3-5 minutes.
- Dip in acid alcohol – 1dip.
- Wash in water for 3-5 minutes.
- Conterstain with eosin for 1 – 2 dips.
- Wash in water for 1-2 dips.
- Dip in alcohol – 1dip.
- Blot, dry and mount in DPX.

The tumours were typed according to the WHO classification. The modified Broder's grading was used to grade the tumours. The important microscopic subtypes of squamous cell carcinoma, presence of premalignant lesions were studied.

TABLE – 1 : MODIFIED BRODER'S GRADING SYSTEM[60]

GRADE 1	GRADE 2	GRADE 3
Well differentiated	Moderately differentiated	Poorly differentiated

ISSN: 0975-3583,0976-2833 VOL14, ISSUE 08, 2023

Cytoplasmic keratinisation, keratin pearls, abundant intracellular bridging	Individual cell keratinisation present	Large tumor cells with scant cytoplasm present
Minimal nuclear pleomorphism	Moderate nuclear pleomorphism	Marked nuclear pleomorphism
<2 mitotic figures/ hpf	4 mitotic figures/ hpf	>4 mitotic figures/ hpf

Modified World Health Organization histological Classification of Invasive carcinoma of the uterine cervix[8]

1. Squamous Cell Carcinoma
2. Adenocarcinoma
3. Other Epithelial tumors

All the 54 cases were subjected to IHC study for p16. One of the case of cervical biopsy diagnosed as chronic nonspecific cervicitis with normal epithelium was also included in the study to know the negative association of p16 (control).The polymer based IHC kit of Biocare Medicals was used.

Preparation of reagents

1. Antigen retrieval Buffer

Tri-sodium citrate buffer: pH – 6 to 6.2
Tri-sodium citrate - 2.94 grams
Dissolve in 1000 ml of water.
Adjust pH with 1N Hcl.

2. Wash buffer

Triss Buffered saline: pH 7.4 to 7.6
Triss buffer - 0.6 grams
NaCl - 8 grams
Dissolve in 1000 ml of water.
Adjust pH with 1N Hcl.

Procedure for IHC staining for p16 antibody

- Cut the sections at approximately 2-3 microns.
- Float on to the positive charged slides.
- Incubate for 37o C for one day and further incubate at 58o C for overnight.
- Two changes of xylene of 15 minutes each for deparaffinization.
- Two changes of absolute alcohol of 3 minute each for rehydration.
- One change of 95% alcohol of 3 minute for rehydration.
- One change of 70% alcohol of 3 minute for rehydration.
- Wash in tap water for 10 minutes.
- Rinse in distilled water for 5 minutes.
- Antigen retrieval by heat, using pressure cooker for 4 whistles.
- Cooling of sections to room temperature.
- Rinse in distilled water for 5 minutes.
- Wash in TBS buffer (pH-7.6) two times for 5 minutes each.
- Treatment with peroxide block for 5 minutes to block endogenous peroxidase enzyme.
- Wash in TBS buffer (pH-7.6) three times for 5 minutes each.
- Treatment with sniper proten (power) block for 10 minutes to block non-specific reaction with the other tissue.

ISSN: 0975-3583,0976-2833 VOL14, ISSUE 08, 2023

- Drain the excess block.
- Wash in TBS buffer (pH-7.6) three times for 5 minutes each.
- Treatment with primary antibody for p16 for 1 hours to identify the tumour markers by antigen-antibody reaction.
- Wash in TBS buffer (pH-7.6) three times for 5 minutes each.
- Treatment with MACH1 Mouse Probe enhancer for 15 minutes.
- Wash in TBS buffer (pH-7.6) three times for 5 minutes each.
- Treatment with MACH1 HRP polymer (secondary antibody) for 30 minutes to elongate chain and also label the enzyme.
- Wash in TBS buffer (pH-7.6) three times for 5 minutes each.
- Treatment with Betazoid DAB Chromogen working solution for 5 minutes to give brown colour to antigens.
- Wash in TBS buffer (pH-7.6) three times for 5 minutes each.
- Wash in tap water for 5 minutes.
- Counter stain with Harris haematoxylin for 1 minute.
- Wash in tap water for 5 minutes to wash away excess stain.
- Two changes of xylene for 15 minutes each.
- Two changes of absolute alcohol for 1 minute each for dehydration.
- One change of 90% alcohol of 2 minutes for dehydration
- Two changes of absolute alcohol of 2 minutes each for dehydration.
- Clearing with xylene for two minutes.
- Mount with DPX.

ASSESSMENT OF EXPRESSION OF P16

Nuclear staining was assessed based on intensity, number, and percentage of positive cells.

Table – 2 : Percentage Of Positivity Of P16 Staining61

Percentage of cells showing p16 positivity (in 10 hpf)	Grade
0 to 10%	1
11% to 50%	2
51% to 80%	3
>80%	4

Table – 3 : Grading of intensity of p16 staining pattern61

Staining pattern	Grade of Intensity
No visible staining	0
Weak staining	1+
Moderate staining	2+
Strong staining	3+

The score of p16 was correlated with clinico-pathological parameters.

Statistical analysis

Data was entered in excel sheet and analysed using SPSS software. Characteristics were summarized descriptively, with meanSD used for continuous variables and number and percentage used for categorical data. Chi-square test was used for association between two variables.

ISSN: 0975-3583,0976-2833 VOL14, ISSUE 08, 2023

The formula for the chi-square statistic used in the chi square test is:

$$\chi_c^2 = \sum \frac{(O_i - E_i)^2}{E_i}$$

The subscript "c" are the degrees of freedom. "O" is observed value and E is expected value. C= (number of rows-1)* (number of columns-1).

In cases of more than 30% cell frequency <5, Freeman-Halton Fisher exact test was employed to determine the significance of differences between groups for categorical data.

If the p-value was < 0.05, then the results were considered to be statistically significant otherwise it was considered as not statistically significant. Data were analyzed using SPSS software v.23.0 and Microsoft office 2007.

SAMPLE SIZE CALCULATION

With 95% confidence level, incidence of cervical HPV infection as 7.9% and margin of error of ±6.2%55, a sample size of 54 subjects will allow the study to determine the "p16 expression in premalignant and malignant lesions of uterine cervix" with finite population correction.

By using the formula:

$$n = \frac{z2p(1-p)}{d2}$$

where

Z= z statistic at 5% level of significance

d is margin of error

p is anticipated prevalence rate (50%)

Type of study

Prospective cross-sectional study

RESULTS

In the present study conducted in the department of pathology, MIMS, Mandya, 54 cases of cervical biopsy/hysterectomy were evaluated from January 2017 to June 2018. Of the 54 cases, 49 were cervical biopsies and 5 were of hysterectomies done for other gynaecologic causes.

In the present study, the age of patients of premalignant and malignant lesions of cervix ranged from 30 to 80 years with a mean age of 52 years. The peak incidence of carcinoma of cervix was seen in the fifth decade. (Table 4,5. Figure3).

TABLE 4: DISTRIBUTION OF CASES ACCORDING TO AGE

AGE (YRS)	N	%
21-30	2	3.7
31-40	10	18.5
41-50	14	25.9
51-60	15	27.8
61-70	12	22.2
71-80	1	1.9
Total	54	100

TABLE 5: MINIMUM, MAXIMUM & MEAN AGE PRESENTATION OF PATIENTS

	Minimum	Maximum	Mean	SD
AGE (YRS)	30	80	52.7	11.9

In the present study, all the cases were married and had child birth. Of the 54 cases, 17 patients (31.5%) were of parity 2 to 3, 36 patients (66.7%) were of parity 4 to 5 and 1 patient (1.9%) were of parity more than 5. Most women had parity of 4 to 5 (66.7%). (Table 6)

ISSN: 0975-3583,0976-2833 VOL14, ISSUE 08, 2023

TABLE 6: DISTRIBUTION OF CASES ACCORDING TO PARITY

PARITY	N	%
2-3	17	31.5
4-5	36	66.7
>5	1	1.9
Total	54	100

In the present study, 35 (64.8%) are post-menopausal women and 19 (35.2%) patients are pre-menopausal. (Table 7)

TABLE 7: DISTRIBUTION OF CASES ACCORDING TO MENOPAUSE

MENOPAUSE	N	%
YES	35	64.8
NO	19	35.2
Total	54	100

In the present study of the 54 cases of lesions cervix, 42 cases (77.8%) presented with white discharge per vagina (WDPV). (Table 8,)

TABLE 8 : DISTRIBUTION OF CASES ACCORDING TO WHITE DISCHARGE PER VAGINA

WHITE DISCHARGE PER VAGINA	N	%
YES	42	77.8
NO	12	22.2
Total	54	100

In the present study, 19 (35.2%) patients presented with low abdominal pain (Table 9)

TABLE 9 : DISTRIBUTION OF CASES ACCORDING TO LOW ABDOMINAL PAIN

LOW ABDOMINAL PAIN	N	%
ABSENT	35	64.8
PRESENT	19	35.2
Total	54	100

In the present study, of the 54 cases of premalignant lesions and carcinoma cervix, 6 cases (11.1%) presented with contact bleeding (CB), 8 cases (14.8%) presented with intermenstrual bleeding, 2 cases (3.7%) presented with menorrhagia , 12 cases (22.2%) presented with mass per vagina 3 case (5.6%) presented with mass per vagina and intermenstrual bleeding, 4 cases (7.4%) presented with mass per vagina and postmenoausal bleeding and 14 (25.9%) presented with postmenopausal bleeding. The most common clinical presentation in pre-menopausal women was WDPV and intermenstrual bleeding and in postmenopausal women it was PMB and WDPV. (Table 10,)

TABLE 10: DISTRIBUTION OF CASES ACCORDING TO MENSTRUAL HISTORY

MENSTRUAL HISTORY	N	%
CONTACT BLEEDING	6	11.1
INTER MENSTRUAL BLEEDING	8	14.8
MENORRHAGIA	2	3.7
MASS PER VAGINA	12	22.2
MPV+IMB	3	5.6
MPV+PMB	4	7.4
PMB	14	25.9
NA	5	9.3
Total	54	100

Family history

In the present study, none of the cases had positive family history of carcinoma cervix.

ISSN: 0975-3583,0976-2833 VOL14, ISSUE 08, 2023

Past history

In the present study, none of the cases had past history of CIN II/III and cervical carcinoma. i.e. all cases were of primary malignancy.

Specimen type

In the present study, out of 54 cases of cervical lesions, 49 cases (90.7%) were of cervical biopsy and 5 cases (9.3%) were of hysterectomy.(Table 11,)

TABLE 11: DISTRIBUTION OF CASES ACCORDING TO SPECIMEN TYPE

SPECIMEN TYPE	N	%
BIOPSY	49	90.7
HYSTERECTOMY	5	9.3
Total	54	100

Among the 5 hysterectomy specimens of carcinoma cervix, 3 cases (5.6 %) was of Stage IA, 1 case (1.9%) were of Stage IIa, and 1 case (1.9%) were of Stage III. Clinical stage was not mentioned in rest of cases. (Table 12)

TABLE 12: DISTRIBUTION OF CASES ACCORDING TO CLINICAL (FIGO) STAGING

CLINICAL (FIGO) STAGING	N	%
I A	3	5.6
II A	1	1.9
III	1	1.9
NA	49	90.7
Total	54	100

MICROSCOPIC EXAMINATION

The study found that 46 cases of premalignant and malignant lesions of cervix were squamous cell carcinoma, 3 cases of CIN III, 1 case of adenocarcinoma, 1 case of adenosquamous Ca, 1 case of CIN I, 1 case of CIN II, and 1 case of microinvasive SCC.

TABLE 13 : DISTRIBUTION OF CASES ACCORDING TO MICROSCOPIC FINDINGS

MICROSCOPIC FINDINGS	N	%
AC	1	1.9
ADSCC	1	1.9
CIN I	1	1.9
CIN II	1	1.9
CIN III	3	5.5
MISCC	1	1.9
SCC	46	85.2
Total	54	100

The 47 cases of various carcinoma of cervix were well differentiated (Grade I), moderately differentiated (Grade II) and poorly differentiated (Grade III).

TABLE14: DISTRIBUTION OF CASES ACCORDING TO MODIFIED BRODER'S GRADING

BRODER'S GRADING	N	%
I	6	11.1
II	34	63
III	7	13
NA	7	13
Total	54	100

SCC cases were divided into LCNK, LCK and small cell types, with 13 (24.1%) being large cell keratinising and 34 (63%) being non-keratinising.

ISSN: 0975-3583,0976-2833 VOL14, ISSUE 08, 2023

TABLE 15 : DISTRIBUTION OF CASES ACCORDING TO HISTOLOGIC TYPE SCC

HISTOLOGIC TYPE SCC	N	%
LARGE CELL KERATINIZING	13	24.1
LARGE CELL NONKERATINIZING	34	63
NA	7	13
Total	54	100

Multifocal invasion was seen in 38 cases (70.4%), while unifocal invasion was seen in 10.

TABLE 16: DISTRIBUTION OF CASES ACCORDING TO FOCUS OF INVASION

FOCUS OF INVASION	N	%
MULTIFOCAL	**38**	**70.4**
UNIFOCAL	**10**	**18.5**
NA	**6**	**11.1**
Total	**54**	**100**

P16 expression was studied in all 54 cases of carcinoma cervix, with 14 cases (25.9%) having p16 grade between 1-2 and 40 cases (74.1%) having p16 grade between 3-4. The mean grade was 3.1.

TABLE 17 : DISTRIBUTION OF CASES ACCORDING TO p16 GRADE

p16 GRADE	N	%
1-2	**14**	**25.9**
3-4	**40**	**74.1**
Total	**54**	**100**

.

TABLE 18 : MEAN VALUE OF p16 GRADE

	Minimum	Maximum	Mean	SD
p16 GRADE	**1**	**4**	**3.1**	**0.8**

The intensity of p16 score was between 1-2 for 40 cases (74.1%) and strong intensity between 3-4 was observed for 14 cases (25.9%). The mean intensity score was 2.1 +/-0.6.

TABLE 19 : DISTRIBUTION OF CASES ACCORDING TO p16 INTENSITY

p16 INTENSITY	N	%
1-2	**40**	**74.1**
3-4	**14**	**25.9**
Total	**54**	**100**

TABLE 20 : MEAN VALUE OF p16 INTENSITY

	Minimum	Maximum	Mean	SD
p16 INTENSITY	**1**	**3**	**2.1**	**0.6**

The study found that 4 cases (28.6%) in the 4th decade and 4 cases (28.6%) in the 5th decade showed p16 grading between 1-2, 11 cases (27.5%) in the 5th decade and 10 cases (25%) in the 4th decade showed p16 grade between 3-4, and 9 cases (22.5%) of the 6th decade were in 3-4 grading. No statistically significant association was noted between p16 grade and age (p value=0.945).

ISSN: 0975-3583,0976-2833 VOL14, ISSUE 08, 2023

TABLE 21 : DISTRIBUTION OF p16 GRADE ACCORDING TO AGE

AGE (YRS)	p16 GRADE				p value
	1-2		3-4		
	N	%	N	%	
21-30	1	7.1%	1	2.5%	0.945
31-40	2	14.3%	8	20.0%	
41-50	4	28.6%	10	25.0%	
51-60	4	28.6%	11	27.5%	
61-70	3	21.4%	9	22.5%	
71-80	0	0.0%	1	2.5%	
Total	14	100.0%	40	100.0%	

Out of 49 biopsy specimens of premalignant lesions and carcinoma of cervix, 38 cases (95%) had p16 grade between 3-4 and 11 cases (78.6%). 3 out of 5 hysterctomy cases (21.4%) showed 1-2 p16 grade and 2 cases (5%) showed 3-4 grade. The p value was 0.068.

TABLE 22 : DISTRIBUTION OF P16 GRADE ACCORDING TO SPECIMEN TYPE

SPECIMEN TYPE	P16 GRADE				p value
	1-2		3-4		
	N	%	N	%	
BIOPSY	11	78.6%	38	95.0%	0.068
HYSTERECTOMY	3	21.4%	2	5.0%	
Total	14	100.0%	40	100.0%	

The grade of p16 positivity was correlating with the clinical (FIGO) stage of 5 hysterectomy cases, with 1 case (7.1%) of stage IA showing grade 2 positivity, 2 cases of stage IA showing grade 3 and 4 positivity, and 1 case (7.1%%) of stage III showing grade 1 positivity. No statistically significant association was noted between clinical stage and p16 positivity grade (p value=0.106).

TABLE 23 : DISTRIBUTION OF p16 GRADE ACCORDING TO (FIGO) STAGING

FIGO STAGING	p16 GRADE				p value
	1-2		3-4		
	N	%	N	%	
I A	1	7.1%	2	5.0%	0.106
II A	1	7.1%	0	0.0%	
III	1	7.1%	0	0.0%	
NA	11	78.6%	38	95.0%	
Total	14	100.0%	40	100.0%	

The Modified Broder's grade of p16 positivity was found to correlate with 1/6 cases (7.1%) of well differentiated carcinoma, 5/6 cases (12.5%) of moderately differentiated carcinoma, 7 cases (50%) of grade 1-2 positivity, 27 cases (67.5%) of grade 3-4 positivity, 3/7 cases (21.4%) of poorly differentiated carcinoma showed grade2 positivity, and 4 cases of poorly differentiated carcinoma showed p16 grade positivity between 3-4. No statistically significant relation was noted between Broder's grade and p16 positivity grade (p value=00413).

TABLE 24 : DISTRIBUTION OF p16 GRADE ACCORDING TO MODIFIED BRODER'S GRADING

MODIFIED BRODER'S GRADING	p16 GRADE				p value
	1-2		3-4		
	N	%	N	%	
I	1	7.1%	5	12.5%	0.413
II	7	50.0%	27	67.5%	

ISSN: 0975-3583,0976-2833 VOL14, ISSUE 08, 2023

III	**3**	**21.4%**	**4**	**10.0%**	
NA	**3**	**21.4%**	**4**	**10.0%**	
Total	**14**	**100.0%**	**40**	**100.0%**	

The most important details in this text are the correlation of p16 positivity with age of 54 cases of premalignant and malignant lesions of cervix. In the 2nd decade, 1 out of 14 cases (25%) showed medium score, 7 out of 10 cases (24.1%) showed medium score and 3/10 cases (14.3%) showed high score. In the 4th decade, 1 out of 14 cases (25%) showed low score, 5/14 cases (17.2%) showed medium score and 8/14 cases (38.1%) showed high score. In the 5th decade, 2/16 cases (50%) showed low score, 8/16 cases (27.6%) showed medium score and 5/16 cases (23.8%) showed high score. In the 6th decade, 1/12 case (25%) showed low score, 7/12 (24.1%) cases showed medium score and 4/12 (19%) showed high score.

The maximum cases were in the 5th decade. The p value was 0.852 which was not statistically significant.

TABLE 25 : DISTRIBUTION OF p16 SCORE ACCORDING TO AGE

AGE (YRS)	p16 SCORE						p value
	1-3		4-5		6-7		
	N	%	N	%	N	%	
21-30	**0**	**0.0%**	**1**	**3.4%**	**1**	**4.8%**	
31-40	**0**	**0.0%**	**7**	**24.1%**	**3**	**14.3%**	
41-50	**1**	**25.0%**	**5**	**17.2%**	**8**	**38.1%**	
51-60	**2**	**50.0%**	**8**	**27.6%**	**5**	**23.8%**	**0.852**
61-70	**1**	**25.0%**	**7**	**24.1%**	**4**	**19.0%**	
71-80	**0**	**0.0%**	**1**	**3.4%**	**0**	**0.0%**	
Total	**4**	**100.0%**	**29**	**100.0%**	**21**	**100.0%**	

The study found that in 17 patients with parity between 2-3, a low p16 score (1-3) was seen in 2 (50%), a moderate p16 score (4-5) was seen in 7 (24.1%) and a high p16 score (6-7) was seen in 8 (38.1%) respectively. In 36 cases with parity of 4 to 5, a high p16 score was seen in 13 (61.9%), a moderate p16 score was seen in 21 (72.4%) and a low p16 score was seen in 2 (50%). This difference was not statistically significant (p value=0.65).

TABLE 26 : DISTRIBUTION OF p16 SCORE ACCORDING TO PARITY

PARITY	p16 SCORE						p value
	1-3		4-5		6-7		
	N	%	N	%	N	%	
2-3	2	50.0%	7	24.1%	8	38.1%	
4-5	2	50.0%	21	72.4%	13	61.9%	0.65
>5	0	0.0%	1	3.4%	0	0.0%	
Total	4	100.0%	29	100.0%	21	100.0%	

Out of 49 biopsy specimens, 3 (75%) showed low p16 score, 26 (89.7%) showed moderate p16 score and 20 (95.2%) showed high p16 score. Out of 5 hysterectomy cases of cervical carcinoma, 1 (25%) showed low score, 3 (10.3%) showed moderate score and 1 (4.8%) showed high score. This difference was not statistically significant (p value=0.422).

TABLE 27 : DISTRIBUTION OF p16 SCORE ACCORDING TO SPECIMEN TYPE

SPECIMEN TYPE	p16 SCORE						p value
	1-3		4-5		6-7		
	N	%	N	%	N	%	
BIOPSY	3	75.0%	26	89.7%	20	95.2%	0.422
HYSTERECTOMY	1	25.0%	3	10.3%	1	4.8%	

ISSN: 0975-3583,0976-2833 VOL14, ISSUE 08, 2023

Total	4	100.0%	29	100.0%	21	100.0%	

Modified Broder's grading showed that 1 out of 6 cases (25%) of well differentiated carcinoma showed low score of 1-3, 1 out of 6 cases (3.4%) showed moderate score and 4 out of 6 cases (19%) showed high score of p16. Out of 34 cases of moderately differentiated carcinoma, 21 cases (72.4%) showed a moderate score and 13 cases (61.9%) showed high score. Out of 7 cases of poorly differentiated carcinoma, 1 case (25%) showed low score, 4 cases (13.8%) showed moderate score and 2 cases (9.5%) showed high score. No statistically significant relation was noted between Broder's grade and p16 positivity.

TABLE 28 : DISTRIBUTION OF P16 SCORE ACCORDING TO BRODER'S GRADING

BRODER'S GRADING	p16 SCORE						p value
	1-3		4-5		6-7		
	N	%	N	%	N	%	
I	1	25.0%	1	3.4%	4	19.0%	0.072
II	0	0.0%	21	72.4%	13	61.9%	
III	1	25.0%	4	13.8%	2	9.5%	
NA	2	50.0%	3	10.3%	2	9.5%	
Total	4	100.0%	29	100.0%	21	100.0%	

The study found that 1 out of 3 cases of clinical stage IA showed low score, 1 out of 3 cases showed moderate score, and 1 out of 3 cases showed high score. One case (3.4%) of clinical stage IIA showed moderate score between 4-5 and one case of clinical stage III showed moderate score of p16 positivity. No statistical significant association was noted between clinical stage and p16 score.

TABLE 29 : DISTRIBUTION OF p16 SCORE ACCORDING TO FIGO STAGING

CLINICAL (FIGO) STAGING	p16 SCORE						p value
	1-3		4-5		6-7		
	N	%	N	%	N	%	
I A	1	25.0%	1	3.4%	1	4.8%	0.562
II A	0	0.0%	1	3.4%	0	0.0%	
III	0	0.0%	1	3.4%	0	0.0%	
NA	3	75.0%	26	89.7%	20	95.2%	
Total	4	100.0%	29	100.0%	21	100.0%	

The study found that 54 cases of premalignant lesions and carcinoma of cervix had over expression of p16, with one case (4.8%) of adenocarcinoma showing high p16 positivity score between 6-7, one case (4.8%) of adenosquamous carcinoma showing high score, one case (25%) of CIN I showing low score of 1-3, one case (3.4%) of CIN II showing moderate score between 3-5, one case of CIN III (25%) showing low score and two cases (6.8%) showing moderate score. Out of 46 cases of invasive squamous cell carcinoma, 1 case (25%) showed low score, 26 cases (89.7%) showed moderate score and 19 cases (90.5%) showed high score between 6-7 for p16 positivity. A statistically significant association was noted between microscopic type and p16 positivity (p value=0.001).

TABLE 30 : DISTRIBUTION OF p16 SCORE ACCORDING TO MICROSCOPIC FINDINGS

MICROSCOPIC FINDINGS	p16 SCORE						p value
	1-3		4-5		6-7		
	N	%	N	%	N	%	
AC	**0**	**0.0%**	**0**	**0.0%**	**1**	**4.8%**	**0.001***
ADSCC	**0**	**0.0%**	**0**	**0.0%**	**1**	**4.8%**	
CIN I	**1**	**25.0%**	**0**	**0.0%**	**0**	**0.0%**	
CIN II	**0**	**0.0%**	**1**	**3.4%**	**0**	**0.0%**	

ISSN: 0975-3583,0976-2833 VOL14, ISSUE 08, 2023

CIN III	**1**	**25.0%**	**2**	**6.8%**	**0**	**0.0%**	
MISCC	**1**	**25.0%**	**0**	**0.0%**	**0**	**0.0%**	
SCC	**1**	**25.0%**	**26**	**89.7%**	**19**	**90.5%**	
Total	**4**	**100.0%**	**29**	**100.0%**	**21**	**100.0%**	

Note: * significant at 5% level of significance (p<0.05)

DISCUSSION:

The mean age of patients with carcinoma cervix was 52 11 years, with the peak incidence occurring in the 5th decade. This is similar to other studies (40 11.5 years, 51-60 years, and 58.9 years).

This study found that most patients of carcinoma cervix were of parity 4-5, which is consistent with the study done by Kava51 S et al in Delhi (3 to 5 parity).

In the present study, the most common clinical presentation in pre-menopausal women was WDPV and intermenstrual bleeding and in postmenopausal women it was PMB and WDPV. (Table 10)

Of the 54 cases of carcinoma cervix majority were squamous cell carcinoma (85.2%) followed by 2 cases of CIN III (3.7%), 1 case each of adenocarcinome (1.9%), 1 case of adenosquamous cell carcinoma (1.9%), 1 case of CIN I (1.9%), 1 case of CIN II (1.9%). This finding is in concordance with Geok Chin Tan et al52, Ruchi G et al 44, Focchi et al 46, Mary D et al59, Mood NI et al61(70%), Shwetha k et al63(70%).

In our study most of the carcinoma of cervix were moderately differentiated (63%), similar to the study done by Rasheed MA62 (55%). In the study done by Klaes et al40, Chaloob MK et al58 and Kishore V55, the most common grade was moderately differentiated carcinoma (51%, 71% and 46% respectively). (Table 31).

TABLE 31: TABLE SHOWING COMPARISON OF BRODER'S GRADE BETWEEN VARIOUS STUDIES

Study name	Grade 1	Grade 2	Grade 3
RasheedMA[62](2010)	**03**	**25**	**12**
Klaes et al[40](2001)	**06**	**21**	**12**
Kishore V[55](2013)	**11**	**24**	**12**
Chaloob MK[58](2016)	**9**	**28**	**16**
Present Study(2016)	**6**	**34**	**7**

Of the 47 cases of SCC, most of the cases were of large cell non-keratinising subtype (63%) followed by large cell keratinising subtype (24.1%). This finding is similar to the studies done by Rasheed MM et al62 (LCNK-50%) , Chaloob MK et al 58 (52%). (Table 32)

TABLE 32: TABLE SHOWING COMPARISON OF HISTOLOGIC TYPE SCC IN VARIOUS STUDIES

STUDY	LCNK	LCK
Rasheed MM[62] et al (2010)	**10**	**6**
Chaloob MK[58] et al (2016)	**38**	**20**
Present Study (2016)	**34**	**13**

P16 expression was assessed in 54 cases of premalignant and malignant lesions of uterine cervix, including CIN I, II, III, microinvasive Ca, Adenocarcinoma, adenosquamous carcinoma and invasive squamous cell carcinoma.

In the present study, out of the 54 cases, 17 patients (31.5%) were of parity 2 to 3, 36 patients (66.7%) were of parity 4 to 5 and 1 patient (1.9%) were of parity more than 5. Most women had parity of 4 to 5 (66.7%).

P16 positivity was found to increase in women with high parity, but no statistically significant association was observed between parity and p53 score (p=0.65). This suggests that expression of p53 increased in women with high parity, but no statistically significant association was observed between parity and p53 score.(Table 26)

ISSN: 0975-3583,0976-2833 VOL14, ISSUE 08, 2023

Postmenopausal women with carcinoma cervix had higher p16 overexpression than premenopausal women, with 68% having higher levels than premenopausal women. Magar SLet al56 also found higher levels of p16 over expression in postmenopausal women.

p16 (ink4a) expression directly reflects infection with high risk HPV in cervical lesions and can add a significant diagnostic accuracy in the evaluation of CIN and SCC.

The p16 was expressed in 100 % of the cases of carcinoma cervix in our study. The p16 expression in various studies ranged from 25.2% to 87.5%. The varying range in different studies may be because of the fixation and AR methods. In various studies done by Godoy AEG et al41(p=0.001), Gupta R et al44 (p=0.000), Krishnappa P et al54 (p=0.05), Ozgul N et al50(0.000), Dordevic B et al53. The sensitivity of p16 positivity is high in cervical carcinoma. (Table 33).

TABLE 33 : TABLE SHOWING INCIDENCE OF p16 IN VARIOUS STUDIES

STUDY (YEAR)	INCIDENCE OF p16 EXPRESSION (%)
Murphy[66] et al (2002)	99%
Aghoff[42] et al (2003)	92%
Godoy AEG[41] (2003)	94%
Gupta R[44] (2004)	95%
Focchi[46]et al (2007)	100%
Ozgul N[50] et al (2008)	100%
Geok CT[52] et al (2010)	98.6%
Dordevic B[53] et al (2011)	100%
Krishnappa P[54] et al (2012)	100%
Kishore V[55] (2013)	75%
Chaloob MK[58] (2016)	94.3%
Present Study (2016)	100%

Our study found that 74.1% of cases showed p16 positivity in more than 50% of tumor cells. Studies by Shwetha K et al63, Godoy AEG et al 41 and Kishore V55 also showed that significant over expression of p16INK4A was observed in carcinoma cervix and with increasing severity of cervical dysplasias, the p16INK4A expression increased progressively. The aggressiveness of the tumour is more when more >30% positivity is seen in tumour nuclei.

Our study found a high percentage of p16 positivity in the early and late stages of cervical carcinoma, with 100% positivity. Chaloob et al58 found p16 expression in 61% cases of clinical stage I, 62% cases of stage II and 87% cases of clinical stage III. It is possible that p16 expression may increase in the more advanced stages of cervical carcinoma due to increased abnormality in control of p16 expression or degradation.

Our study found that all 46 cases (85.2%) of invasive squamous cell carcinoma showed 100% p16 positivity, similar to other studies.

Our study found p16 postivity in single cases of CIN I, CIN II, CIN III and Ca-in-situ. In various studies, variable positivity has been detected in premalignant lesions. Kishore V55's study showed positivity in 25% cases of CIN1, 50% cases of CIN 2 and 75% cases of CIN3. Table 34 lists the p16 expression in various premalignant and malignant lesions.

TABLE 34: p16 EXPRESSION IN VARIOUS CERVICAL NEOPLAMS IN VARIOUS STUDIES(%)

STUDY	CIN I	CIN II	CIN III	SCC
Focchi[46] et al	91%	100%	NOT DONE	100%
Aghoff[42] et al	57%	75%	91%	92%

ISSN: 0975-3583,0976-2833 VOL14, ISSUE 08, 2023

Tringler[65] et al	**72%**	**100%**	**NOT DONE**	**100%**
Gupta R[44]	**50%**	**60%**	**70%**	**95%**
Murphy[66] et al	**100%**	**100%**	**98%**	**100%**
Present study	**100%**	**100%**	**100%**	**100%**

Above various studies impression showed that the p16 expression increases with increasing severity and grades of cervical neoplasms.

Chaloob et al58 found that 66.7% cases of adenocarcinoma showed high p16 score, while 2 cases (66.7%) of ADSCC were positive for p16 expression. This indicates strong positivity and intensity for p16.

This study found that p16 positivity was seen in all grades of cervical carcinoma, with moderate scores in 72.4% and high scores in 61.9% cases. Chaloob et al58 found high positivity for Grade III SCC (68.8%) followed by grade II and grade I, but no statistically significant relation was found between the Broder's grade and p16 positivity (p value=0.072).

Magar SL56's study showed that p16 positivity was found in 31/46 cases of squamous cell carcinoma cervix, 28.57% (2/7) cases of CIN1, 44.44% (4/9) cases of CIN2, and 71% (7/10) cases of CIN3. P16 expression increased with increasing grade of CIN and also in SCC. Most of the SCC showed p16 INK4A expression, with p16 INK4A staining confined to the lower 1/3 of epithelium in CIN1. In CIN 2, p16 INK4A staining was confined to the lower 2/3 of the epithelium, and in CIN 3, the dysplastic epithelium showed full thickness p16 staining. Out of 20 cases of squamous carcinomas, 2 cases were negative for p16 INK4A expression and 18 cases exhibited variable degree of expression for p16 INK4A. 2 cases of CIN 1, 4 cases of CIN 2, and 7 cases of CIN 3 exhibited variable degree of expression for p16.

CONCLUSION:

This study evaluated the p16 expression in 54 cases of various types of carcinoma cervix. A correlation was found between the p16 expression subtypes of CIN and invasive carcinoma of cervix. The p16 positivity was assessed by a semi quantitative method, with the number of cells showing positivity (grade) and the intensity of p16 being studied.

Our data showed a statistically significant correlation between p16 score (p value=0.001) and microscopic type of carcinoma cervix. The p16 positivity increased with age and parity, but the difference was not statistically significant. Similar increases of grade and score of p16 expression were seen with increase in stage and grade of tumour, but with no statistical significance. This may be due to the small sample size of our study.

P16 expression was found in premalignant and malignant cervical lesions, with greater and stronger expression in invasive cervical carcinoma and squamous cell carcinomas. This suggests that p16 expression is a late phenomenon in the pathogenesis of cervical cancer and is a powerful prognostic marker.

Studies with large samples of carcinoma cervix and correlating p16 expression with clinicopathological parameters are needed to develop p16 targeted therapy for carcinoma cervix. Further HPV studies and other markers of carcinoma cervix are suggested.

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