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Original Research Article

Role of p16 expression in preneoplastic and neoplastic lesions of cervix

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ABSTRACT

Background: Cancer of the cervix is the leading cause of cancer among women in India. Human papilloma virus plays an important role in the causation of preneoplastic and neoplastic cervical lesions. HPV type-specific oncoproteins interact with cellular regulatory proteins resulting in upregulation of p16, a cyclin dependent kinase inhibitor. This has made p16, a valuable surrogate biomarker of HPV infection, useful in evaluating HPV associated preneoplastic and neoplastic lesions of cervix. The aim of this study was to evaluate p16 expression in preneoplastic and neoplastic cervical lesions.
Methods: A total of 93 specimens diagnosed histopathologically as cervical preneoplasia and neoplasia were included in this prospective study of one year duration. Maximum cases were of Neoplastic lesions followed by preneoplastic lesions. Majority of the neoplastic lesions were Squamous cell carcinoma. Immunohistochemical (IHC) staining for p16 was performed and was scored by percentage positivity and reaction intensity. p16 positivity in neoplastic lesions was significantly ($p<0.0001$) higher than preneoplastic lesions.
Results: Of the 93 cases, 17 (18.28%) were preneoplastic and 76 (81.72%) neoplastic lesions. In the preneoplastic group, 52.94% cases were p16 positive while 47. 06% cases revealed p16 negativity. Among the neoplastic group, 85.53% cases were p16 positive while 14.47% cases were p16 negative.
Conclusions: p16 expression progressively increased with increasing grades of cervical preneoplastic and neoplastic lesions, establishing p16 as a supplementary marker for early diagnosis of cervical cancer.

Keywords: Cervical cancer, p16, HPV, Preneoplastic, Neoplastic

INTRODUCTION

Cancer of the cervix uteri is the 3rd most common cancer among women worldwide and the 2nd leading cause of female cancer in India.[1] There is an established association between certain subtypes of Human Papilloma Virus (HPV), high grade precursor lesions and cervical carcinoma.[2] Nearly 70% of all cervical cancer cases are attributed to only 2 types, HPV 16 and HPV 18.[3] HPV targets nuclei and number of proteins. Alterations of cellular proteins at different levels occur with progression to cancer. One such protein is p16-INK4a (henceforth referred to as p16), an important cell cycle regulatory molecule which is upregulated following HPV infection. This makes it a useful biomarker for detection of HPV infection.[4] The two viral oncoproteins E6 and E7 interact with cell cycle regulatory proteins, namely, p53, a tumor suppressor protein and Rb, Retinoblastoma protein. This binding leads to degradation of p53, that prevents cell cycle arrest or apoptosis. The HPV-E7 oncoprotein binds and inactivates the tumor suppressor protein pRB, which disrupts the cell cycle, increases proliferation, which eventually results in development of carcinoma. Several studies have shown that status of Rb expression significantly influences p16 expression. Functional inactivation of Rb gene by HPV-E7 protein results in overexpression of p16.[5] In the

present study, we have evaluated the p16 expression in preneoplastic and neoplastic cervical lesions.

METHODS

The present study was a cross-sectional, observational study carried out for a period of one year (June 2017 to May 2018) in the Department of Pathology, IGMC, Shimla. Ninety-three specimens (hysterectomy and cervical biopsies) of cervical preneoplastic and neoplastic lesions were included in the study. Patients showing recurrence of cervical malignancies and undergoing treatment and HPV vaccinated patients with cervical malignancies were excluded. In our study we classified cervical dysplasia according to CIN classification and cervical tumors according to WHO classification 2016.[6] Patient consent and ethical clearance from Institutional Ethical Committee were taken.

Immunohistochemistry (IHC)

IHC was done as per the staining protocol using Mouse monoclonal antibody to p16; clone G175-405 (mouse IgG antibody). Two-step process was followed to demonstrate the antigens by IHC. First step involved binding of a primary antibody to the antigen of interest, and in the second step bound antibody was detected by a chromogenic signal from the stained tissues and cells. Case of cervical squamous cell carcinoma with known positivity was used as positive control. For negative control, phosphate buffer was used instead of primary antibody. Interpretation: Percentage positivity of tumor cells and staining intensity were assessed for grading p16 immunoreactivity. Positivity was seen as brown reaction product in the nucleus and/or cytoplasm (Table 1).

Table 1: Percentage of p16 positive cells.

Grade	% positive tumor cells
0	0
1	0-5
2	5-25
3	>25

Case of cervical squamous cell carcinoma with known positivity was used as positive control. For negative control, phosphate buffer was used instead of primary antibody. Interpretation**:** Percentage positivity of tumor cells and staining intensity were assessed for grading p16 immunoreactivity. Positivity was seen as brown reaction product in the nucleus and/or cytoplasm.

Reaction intensity of p16 immunostaining

Intensity was determined according to the mentioned scale 0: Negative-no stained cells, 1: weak-scattered or diffuse, weakly stained cells, 2: moderate-scattered or diffuse, moderately stained cells and 3: strong-all cells stained strongly and diffusely throughout the lesion.

Statistical analysis

The significance of p16 expression was calculated using SPSS software version 21 and Chi-square test was applied. Significance was assumed at $p<0.05$.

RESULTS

Of the 93 cases, 17 (18.28%) were preneoplastic and 76 (81.72%) neoplastic lesions. In the preneoplastic group, 52.94% cases were p16 positive while 47.06% cases revealed p16 negativity. Majority of CIN I cases (70%) were p16 negative in contrast to CIN II and CIN III which showed p16 positivity in 66.67% and 75% cases respectively.

Out of 3 positive cases of CIN I, reaction intensity was mild in 2 cases and moderate in 1 case. Two out of 3 positive cases of CIN II and 1 case of CIN III revealed moderate staining intensity. Strong intensity of staining was observed in 2 cases of CIN III. Among the neoplastic group, 85.53% cases were p16 positive while 14.47% cases were p16 negative.

Majority cases of Squamous cell carcinoma (90.62%) were p16 positive with strong staining intensity in 56.25% and moderate intensity in 34.38% cases. All the three cases of Adenosquamous carcinoma revealed p16 positivity of moderate intensity.

Table 2: P16 expression.

P16 positivity, diagnosis N (%)	0 (0%)	1 (0-5%)	2 (5-25%)	3 (>25%)	Total
CIN I	7 (70)	2 (20)	1 (10)	0	10
CIN II	1 (33.33)	0	2 (66.67)	0	3
CIN III	1 (25)	0	1 (25)	2 (50)	4
Adenoid basal carcinoma	1 (50)	0	1 (50)	0	2
Small cell (neuroendocrine) carcinoma	2 (100)	0	0	0	2
Adeno squamous carcinoma	0	0	3 (100)	0	3
Adenocarcinoma	2 (40)	0	0	3 (60)	5
Squamous cell carcinoma	6 (9.38)	0	13 (20.31)	45 (70.31)	64

Table 3: Reaction intensity of p16 staining in preneoplastic and neoplastic lesions of cervix.

P16 Intensity, Diagnosis N (%)	Negative (0+)	Weak (1+)	Moderate (2+)	Strong (3+)	Total
CIN I	7 (70)	2 (20)	1 (10)	0	10
CIN II	1 (33.33)	0	2 (66.67)	0	3
CIN III	1 (25)	0	1 (25)	2 (50)	4
Adenoid basal carcinoma	1 (50)	0	1 (50)	0	
Small cell carcinoma (neuroendocrine)	2 (100)	0	0	0	2
Adenosquamous carcinoma	0	0	3 (100)	0	3
Adeonocarcinoma	2 (40)	0	0	3 (60)	5
Squamous cell carcinoma	6 (9.38)	0	22 (34.38)	36 (56.25)	64
Total	20 (21.50)	2 (2.15)	30 (32.26)	41 (44.0993)	93

One out of 2 cases of adenoid basal carcinoma showed positive p16 staining of moderate intensity. Both cases of small cell undifferentiated carcinoma were p16 negative. Grading of p16 expression and reaction intensity of p16 staining in preneoplastic and neoplastic lesions was done (Table 2 and 3).

Relation between types of lesions and p16 positivity

In our study, we observed that 89% of the p16 positive specimens were neoplastic.

We also found that the p16 positivity in neoplastic lesions was significantly higher ($p<0.0001$) than p16 positivity in preneoplastic lesions (Table 4).

Table 4: Relation between types of lesions and p16 positivity.

Type of lesions	Negative (N=20)	Positive (N=73)	P value
Preneoplastic	9 (45)	8 (11)	<0.0001
Neoplastic	11 (55)	65 (89)	<0.0001

DISCUSSION

The role of HPV infection in cervical carcinogenesis is well established. Most of the HPV infection is transient, its persistence increases the risk of developing preneoplastic lesions and subsequently cervical cancer. p16, a promising biomarker, has attracted attention as various studies have documented p16 over-expression in HPV infected lesion.[7] The present study was conducted to evaluate the p16 expression in preneoplastic and neoplastic cervical lesions. Among 93 samples evaluated, 17 (18.28%) cases were of preneoplastic lesions and 76 (81.72%) cases were neoplasms. In our study, p16 positivity was seen in 3 (30%) cases of CIN I, 2 cases (66.67%) of CIN II and 3 cases (75%) of CIN III. In CIN I, our findings correlated with the observations of Tan et al and Volgareva et al.[8,9] In cases of CIN II and CIN III, concordance was observed with Gupta et al and Kishore et al.[4,10-15]

p16 positivity in CIN

The lower expression of p16 in low-grade lesions in our study could be due to the infection by low-risk HPV types whose E7 protein has lower affinity for Rb than that of HR-HPV, and hence, the absence of over-expression of p16. Agoff et al reported similar finding.[5] Tan GC explained that the high percentage of negativity of p16 in CIN I could be due to latent infection with low viral load that may be insufficient for p16 expression.[8] In our study we found negative p16 expression in 1 case each of CIN II and CIN III. Similar observations were made by Volgareva et al and Kang et al who correlated this lack of immunoreactivity with promoter region hypermethylation and p16 gene silencing.[9,10] On semi quantitative scoring, over-expression of p16 increased as cervical dysplasia progressed from CIN I (30%) to CIN 3 (75%). These findings are in accordance with Lesnikova et al, Kishore et al.[15-17] p16 has emerged as a useful screening tool for detection of HR-HPV infection in cervical precancerous lesions and neoplasms. Over-expression of p16 has been reported in both cervical squamous and adenocarcinoma. We observed p16 over-expression in 90.62% cases of Squamous cell carcinoma similar to most authors. Six cases of SCC showed negative p16 immuno-expression which is in concordance with the finding of Agoff et al.[5] The possible explanation for the absence of expression in these high grade lesions could be methylation of the p16 promoter resulting in silencing of the p16 gene as reported by Ferreux et al.[11]

Tripathy et al noted p16 promoter hyper-methylation and homozygous deletion in 6.5% and 8.7% samples respectively.[12] Murphy et al had suggested that p16 is not only a diagnostic marker for cervical squamous lesions but also for glandular neoplastic lesions.[13] In the present study, 60% cases of adenocarcinoma were positive for p16 close to the observations of Agoff SN et al (75%) and Mood I et al (75%).[5,14] However Kishore et al found p16 positivity in 100% cases of adenocarcinoma.[15] The present study showed 3 cases of adenosquamous carcinoma which were 100% positive similar to Umar et

al who observed 100% positivity in 2 cases of Adenosquamous carcinoma.[16]

We observed 2 cases of Adenoid basal carcinoma which showed 50% positivity for p16. Both cases of small cell carcinoma were p16 negative similar to findings of Pao CC et al.[17-20] We observed that expression of p16 increased with increasing grades of CIN. In neoplastic lesions, most of the SCC i.e., 45 (70.31%) cases showed Grade 3 p16 positivity. In our study we also found 100% Grade 2 positivity in adenosquamous carcinoma. None of the studies have reported grading of p16 expression in these histologic types. In the neoplastic group, strong staining intensity was noted in majority (56.25%) of SCC followed by moderate intensity in 34.38%. Mood et al found strong intensity in 75% cases and Kumari et al in 100% of their study cohort.[14,19] In our study we observed that p16 positivity in neoplastic lesions was significantly higher than p16 positivity in preneoplastic lesions. The p value was statistically significant ($p<0.0001$) similar to studies done by Gupta et al, Kishore et al, Srivastava et al, Kumari and Vadivelan et al.[4,15,18-20]

Limitations

Our study was limited by a small sample size and lack of HPV detection. Large population-based studies are required to establish p16 as a supplementary marker for early diagnosis of cervical cancer. The attempt for HPV DNA detection studies to validate the utility of p16 for detection of HPV in cervical neoplasm could not be made due to financial constraint.

CONCLUSION

In our prospective study efficacy of p16 as a surrogate marker for HPV infection was assessed. p16 expression progressively increased with increasing grades of cervical preneoplastic and neoplastic lesions. Percentage positivity as well as staining intensity increased with increasing grades. Significant statistical difference in expression of p16 between preneoplastic and neoplastic lesions ($p<0.0001$) was noted.

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