

Original Research Article

P16, Ki67 and P63 staining pattern in squamous metaplasia, CIN and cervical cancer

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ABSTRACT

Background: Persistent infection with Human Papilloma Virus (HPV) has been the main cause of squamous intra epithelial neoplasia which in turn leads to cancer. The incidence is 28.0%. Early identification of dysplasia and malignancy helps early intervention.

Methods: To do Immunohistochemical staining using P16ink4a, Ki 67, and P63 in cervical squamous metaplasia, CIN I, II, III and correlate the H and E features with IHC patterns. Study was carried out in SRM Medical College Hospital and Research centre, Kattankulathur, Tamil Nadu, a descriptive study for a period of 2 years (2012 to 2014) on formalin fixed paraffin embedded tissues from cervix. H and E sections of uterine cervix were categorized into squamous metaplasia, CIN I, CIN II, CIN III and squamous cell carcinoma. 50 representative samples were subjected to Four-micrometer-thick sections and subjected to IHC using PathInsitu, using P16ink4a, Ki67 and P63. Statistics: Using SPSS for windows (V.17). Data expressed by number and percentage. Methods used were Chi square test, Screening test and ROC curve. Statistical significance was 0.05.

Results: P63 has shown to be the best marker out of the three to distinguish the progression of a lesion towards dysplasia and malignancy in cervix. Ki 67 showed a specificity of 84.2% with a negative predictive value of 59.3%, and an ROC curve area of 69.2%. In this study, Ki67 showed lesser sensitivity than that of P63.

Conclusions: P16 identifies HPV 16 infection in uterine cervix. Ki67 and P 63 are helpful in determining the nature of progression of lesion. High expression of Ki 67 indicates a neoplastic progression. P63 may be used to differentiate benign from malignant lesions. P16 with P63 showed good results in predicting the progression of a lesion.

Keywords: CIN, Cancer cervix, Ki67, p16ink4a, p63

INTRODUCTION

Cervical cancer is the third most common cancer among women worldwide.¹ There is an estimated annual global incidence of 500 000 cancers, of which India contributes 1,00,000, i.e., one-fifth of the world burden.² Persistent infection with Human Papilloma Virus (HPV) has been the main cause of squamous intra epithelial neoplasia which in turn may lead to in situ changes and cervical cancer. In order to see the association of HPV infection in cervix, the most common histological changes in cervix

such as squamous metaplasia, CIN 1, 2, 3 and associated with HPV infection expression of three biomarkers P16 ink 4a, Ki67 and P63 were analysed.

HPV infection of any type was associated with a 498-fold increased risk for cervical cancer. The most common HPV types reported in India were (in descending order) HPV-16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68.³⁻⁶ Patients infected with HPV-18 had a higher risk for cervical cancer compared to women infected with HPV-16. The high-risk viruses play a role in the carcinogenic

process by producing two oncoproteins encoded by the viral E6 and E7 genes. These oncoproteins directly inactivate p53 and pRb, respectively, and promote continued cell cycle and DNA synthesis by blocking apoptosis thus favoring viral replication.

P16 ink4a (p16 inhibitor kinase 4a) functions to decelerate the cell cycle and promote cellular aging in response to multiple stress factors such as oncogene activation, telomere erosion, reactive oxygen species, and stalled replication forks. Normally it is inactivated by genetic deletion or by hyper methylation. When induced, p16 binds and inhibits cyclin-dependent kinase 4/6 (CDK4/6) activity, thereby promoting a retinoblastoma (RB)-dependent cell-cycle arrest resulting in over expression of the P16 which over time gets accumulated in the nucleus and cytoplasm of the affected cell and can be detected with the help of immunostains.⁷ P16 activation is a characteristic feature of all emerging cancers, making the p16LUC allele, a sensitive unbiased reporter of neoplastic transformation.⁸ The P16ink4a (CDK inhibitor) is detected by using anti-P16ink4a antibody. The benign squamous epithelium is commonly either completely negative for p16INK4a or show a focal pattern (sporadic or patchy) restricted to the more differentiated intermediate or superficial squamous epithelial cell layers. Wang et al found that the risk for CIN progression is higher in women with p16 protein compared with those without p16 protein.^{9,10}

Ki 67 immunohistochemical marker

Ki67 (MIB 1) is a nuclear protein and is widely accepted as a proliferation marker in pathology. Ki-67 protein is present during all active phases of the cell cycle, but is absent from resting cells (G0), making it an excellent marker for determining the growth fraction of a given cell population and thus determine the degree of neoplasia.¹¹ In the study conducted by Maria Benevolo et al, the sensitivity and specificity for p16INK4A and Ki-67 immunostaining to detect high-grade squamous intraepithelial neoplasia were 95.12% and 73.68%, respectively with a positive predictive value of 79.59%.

P63: immunohistochemical marker

P63 is a homologue of p53, another being p73. P53 plays a well-established role in tumor suppression, whereas p63 and p73 play unique roles in keratinocyte morphogenesis.¹²⁻¹⁴ In cervical carcinoma, p63 is a marker of squamous differentiation preferentially expressed in immature squamous cells and thus useful in diagnosis during early cancer stages. P63 also helps in identifying squamous cell carcinomas of head and neck, lung, cervix, helps in identification of myoepithelial cells in breast cancer.¹⁵ In bladder cancer, it helps in predicting the prognosis. Also expressed in thymomas, non-Hodgkin's lymphoma, basal cell carcinoma, follicular and papillary carcinoma of thyroid.¹⁶

METHODS

Cervical biopsies and hysterectomy cases (n= 130) were reviewed (H and E) during the period of 2012 to 2014 out of which 96 cases were hysterectomies and 34 cervical biopsies. These were categorized into squamous metaplasia, CIN 1, CIN II, CIN III and squamous cell carcinoma. Out of these 50 cases with good tissue representativeness were subjected to immunohistochemistry using P16, Ki 67 and p63. Four micrometer H and E stained slides were cut. Immunohistochemistry was done using positively charged (pathinsitu) slides. The expression of P16, Ki67 and P63 were analyzed individually and also in combination with corresponding H and E. The association of HPV with metaplastic and dysplastic epithelium were recorded and the infectivity were analyzed based on the IHC expression. The progression of the lesion was studied based on relationship of P16 with Ki67 and P16 with P63. The scoring was done individually on all IHC slides based on distribution (as negative, 1+, 2+, 3+), localization (nuclear, cytoplasmic, nucleocytoplasmic) and pattern (diffuse or focal).¹⁷ The data were analyzed using SPSS for windows (V.17) using the statistical methods Chi square test, Screening test and ROC curve. The statistical significance was considered to be 0.05 level.

RESULTS

The samples consisting of squamous metaplasia, dysplasia and squamous cell carcinoma was seen within 40-49 years age group followed by 30-39 years. Out of 50 cases, 43 samples were successfully stained and were available for interpretation based on distribution, localization and pattern of involvement. A score of 0 and 1 was considered to be negative and score of 2 and 3 was considered as positive.

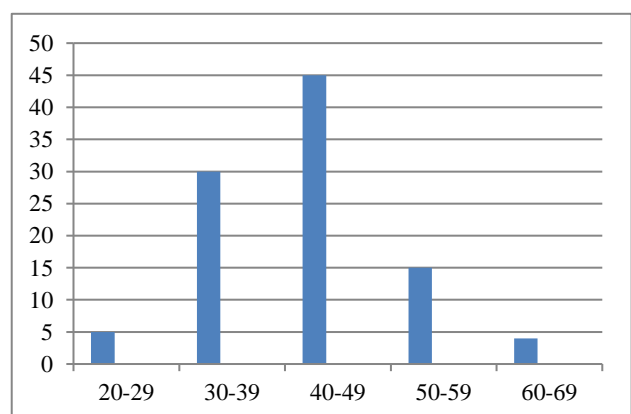


Figure 1: Age distribution of study group.

P 16 expression: In squamous metaplasia, 7/11 (63.6%) cases were positive for P16. Among squamous metaplasia and CIN I group, 4/8 (50%) were positive for P16. In the CIN I group, 1/8 (12.5%) showed P16 positivity.¹⁸ Within

the CIN II group, 3/6 (50%) were positive for P16. CIN III group and Squamous cell carcinoma group, (2/2) and (8/8) showed 100% positivity for P16. This data shows a

strong relationship of P16 ink 4a with squamous metaplasia, CIN II, CIN III and squamous cell carcinoma with a p value < 0.05.¹⁹

Table 1: Cervix-histology on (H and E).

Type of lesion	Frequency	Percent	Valid percent	Cumulative percent
Squamous metaplasia (SM)	94	72.3	72.3	72.3
SM + CIN I	8	6.2	6.2	78.5
CIN I	9	6.9	6.9	85.4
CIN (II + III)	9	6.0	6.0	92.3
SCC	10	7.7	7.7	100.0
Total	130	100.0	100.0	

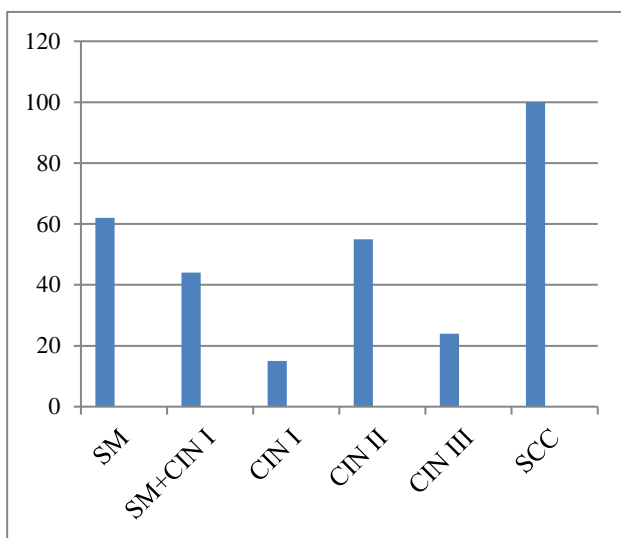


Figure 2: Percentage positivity of P16 ink4a.

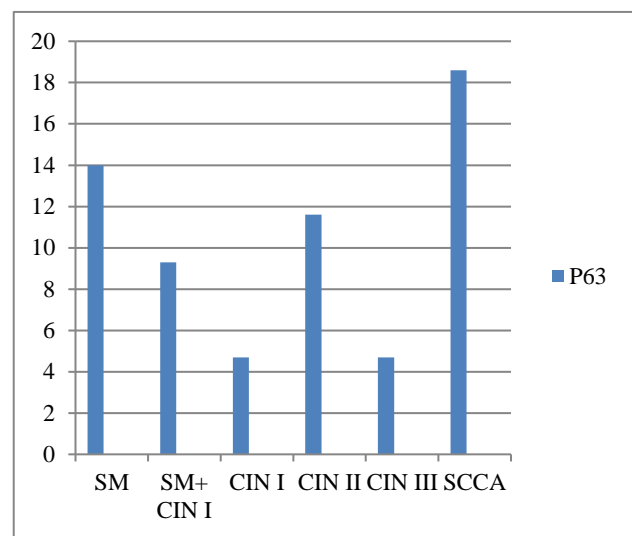


Figure 4: Percentage positivity of P 63.

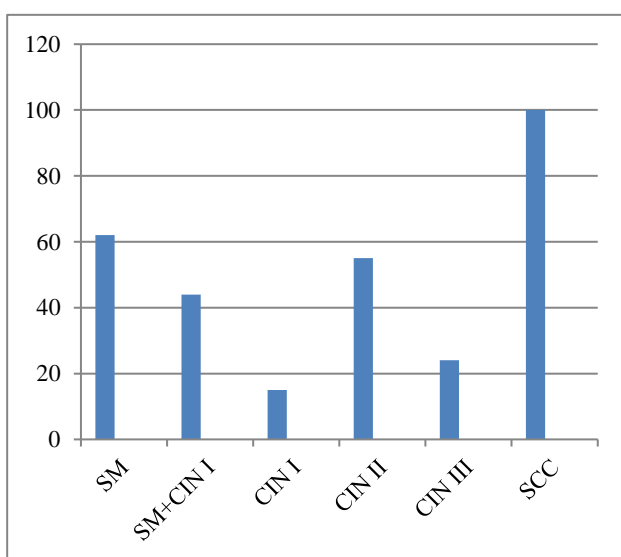


Figure 3: Percentage positivity of Ki 67.

Table 2: P value of P16 (Chi-Square test).

	Value	DF	Asymp. Sig. (2-sided)
Pearson chi-square	115.540	30	0.000
Likelihood ratio	77.737	30	0.000
Linear-by-linear association	0.020	1	0.889
N of valid cases	43		

Ki 67 expression: In squamous metaplasia, Ki 67 was positive in 27.3% of samples. In CIN I with Squamous metaplasia, 12.5% were positive. In CIN I alone showed no positivity. CIN II and III group showed 50% positivity. Overt malignancy showed 100% strong expression.

Thus Ki 67 has strong association with squamous cell carcinoma when compared with the other groups. The p value was 0.000.²⁰

P63 expression: P63 showed a 54.5% positivity in squamous metaplasia. the category squamous metaplasia with CIN I showed 50% positivity. CIN I group alone showed no positivity. CIN II showed 83.3% positivity. CIN III and squamous cell carcinoma showed 100% positivity. The findings suggest a strong relationship between Ki67 and the progression of the lesion with a p value <0.05.²¹ **Squamous metaplasia:** The total number of squamous metaplasia in our study was 16 samples. These were further categorized into: Endocervical glandular metaplasia: Metaplasia of the endocervical glands by squamous cells. (50%) were positive for p16. Ki 67 was negative in all samples although P63 was positive (50%) cases. One sample showed positivity for both the markers P63 and Ki67 (25%). One sample showed immature nucleus which was P63 positive and P16, Ki67 negative. Squamous metaplasia overlying deep into endocervix 25% showed suspicious mild nuclear atypia. The IHC was nonspecific in these. Transitional Zone metaplasia: This group has 2 samples. Both were positive for Ki67 and P63 (100%). One sample was positive for P16 ink 4a (50%).

Table 3: P Value of Ki 67 (Chi-Square test).

	Value	DF	Asymp. Sig. (2 sided)
Pearson chi-square	87.846	25	0.000
Likelihood ratio	63.443	25	0.000
Linear-by-linear association	5.226	1	0.022
N of valid cases	43		

Table 4: Chi- square test.

	Value	DF	Asymp. Sig. (2 sided)
Pearson chi – square	136.80	30	0.000
Likelihood ratio	93.631	30	0.000
Linear-by-linear association	2.849	1	0.091
N of cases			

Comparison of biomarker combinations

P16ink4a with Ki67: In squamous metaplasia, the expression for both P16 and P63 was 9.0%. In squamous metaplasia with CIN I group and in CIN I singly, 12.5% showed positivity in each group. CIN II and III showed 50% positivity for both biomarkers. In overt malignancy, 100% were positive. **P16 with P63:** In squamous metaplasia, 9.1% was positive for both P16 and P63. In squamous metaplasia with CIN I, 25% showed both positivity. CIN I alone showed 12.5% positivity. CIN II, 16.7% were positive for both. In CIN III and squamous cell carcinoma, 100% showed strong expression for both. **Ki67 with P63:** Both positivity, in squamous metaplasia was 18.2 %. Squamous metaplasia with CIN I were 25.0%. CIN I alone was 12.5%. CIN II, 16.7% showed

both expression. CIN III and squamous cell carcinoma, 100% expression of both markers were seen.

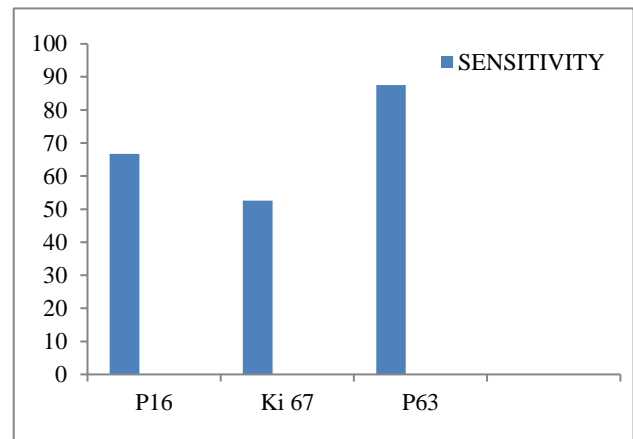


Figure 5: Comparison between the sensitivity between biomarkers.

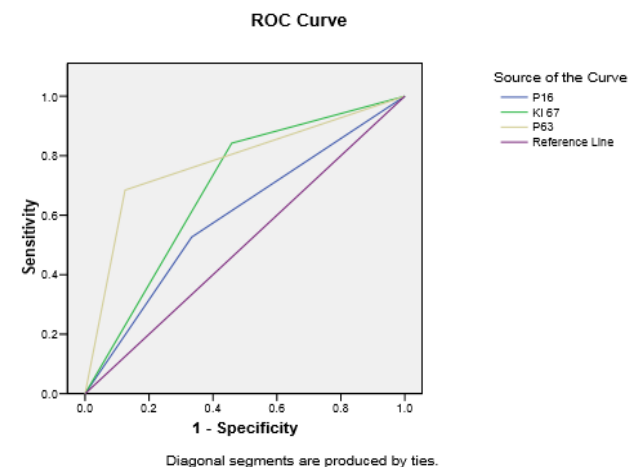


Figure 6: ROC showing the sensitivity of each marker.

Sensitivity and specificity: For calculation of sensitivity and specificity of the biomarkers, Squamous metaplasia and squamous metaplasia with CIN I changes were considered to be benign and CIN I as single and CIN II, III and squamous cell carcinoma as malignant. P63 showed highest sensitivity of 87.5% with a negative predictive value of 81.3% and an area of 78.0 % in the ROC curve.

P63 has shown to be the best marker out of the three to distinguish the progression of a lesion towards dysplasia and malignancy in cervix. Ki 67 showed a specificity of 84.2% with a negative predictive value of 59.3%, and an ROC curve area of 69.2%. In this study, Ki67 showed lesser sensitivity than that of P63.²²⁻²⁵

Scoring: Scoring system was analyzed for all the three biomarkers. The scoring was done based on distribution, localization and pattern of involvement. In this study, the

best method of scoring was the one based on localization (p value of 0.000) and pattern of involvement (p value 0.003).

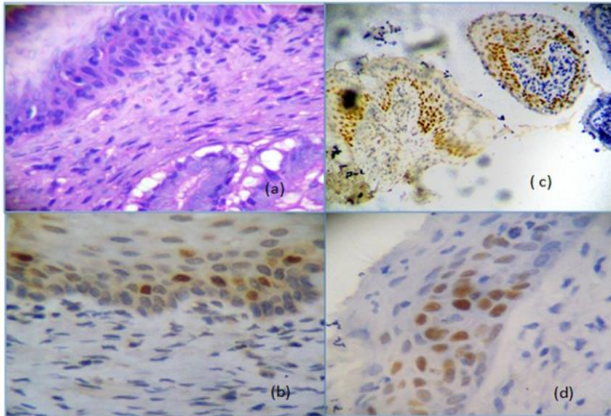


Figure 7: Squamous metaplasia: (a) H and E, (b) P16ink4a, (c) Ki67, (d) P63.

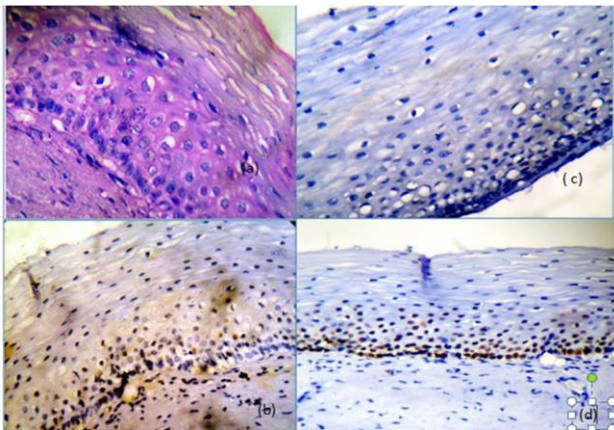


Figure 8: Cervical intraepithelial neoplasia, Type 1: (a) H and E, (b) P16ink4a, (c) Ki67, (d) P63.

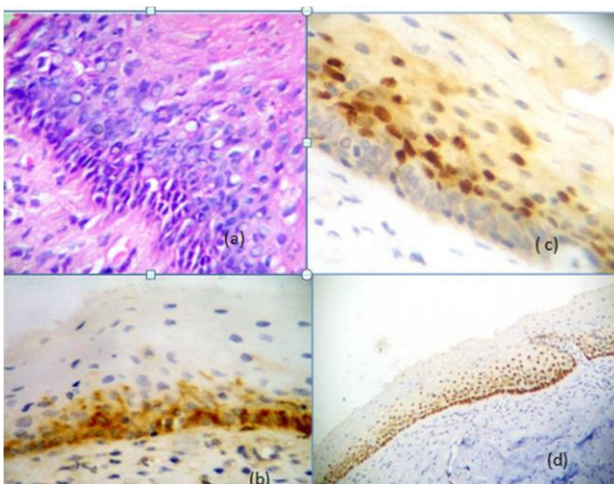


Figure 9: Cervical intraepithelial neoplasia, Type 2: (a) H & E, (b) P16ink4a, (c) Ki67, (d) P63.

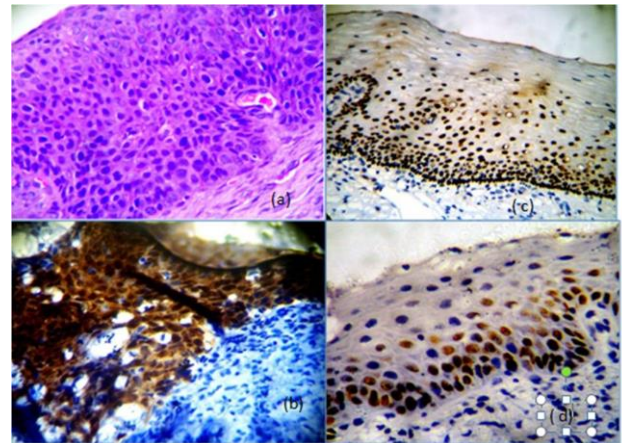


Figure 10: Cervical intraepithelial neoplasia, Type 3: (a) H and E, (b) P16ink4a, (c) Ki67, (d) P63.

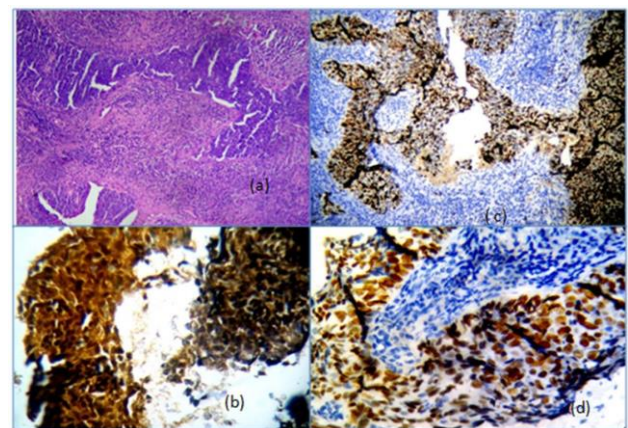


Figure 11: Squamous cell carcinoma: (a) H and E, (b) P16ink4a, (c) Ki67, (d) P63.

DISCUSSION

Cervical cancer is the first cause of cancer in women in the age group 15-45yrs main etiology being young age of onset of sexual intercourse. Early detection of viral infection helps in prophylaxis and prompt treatment. Study by Brown et al showed strong association of HPV infection with cervix and as a potential predisposing factor for the onset of cervical malignancy. Studies by Mary T Galgano et al and Agnieszka K Witkeiwicz et al established P16 ink4a as a good marker for identification of HPV infection in cervix. Ki 67 is an established proliferative marker. In this study, P63 showed good expression in cervix similar to the studies by Bradley J. Quade et al and Su Mi Kim et al. Identifying the host cell in cervix, which when infected by HPV, has the maximum potential to undergo mutation and differentiation causing malignancy, can help in administering early targeted therapy to patients.²¹

P16 expression in this study was found to be 63.6% within squamous metaplasia with a gradual increase from CIN I, II and III, maximum in squamous cell carcinoma,

indicating a possibility of HPV infection starting from endocervical columnar cells resulting in squamous metaplasia within these cells. In the study by Loris Y. Hwang and Yifei Ma et al (n=139) stated that the time taken from initial HPV infection till squamous metaplasia is 4-5 months.²² The overall rate of squamous metaplasia was 4.2% per year and proposed that each 1% metaplastic change per month was associated with a 17% increased risk for subsequent HPV16 incidence. In squamous metaplasia group, the transitional zone and glandular metaplastic cells showed 50% positivity for p16 when compared with the endocervical type indicating that the HPV virus has higher affinity to transitional zone and metaplastic cells of endocervical glands. Moscicki et al concluded active squamous metaplasia as the main risk factor for low grade intraepithelial lesion, rather than the size of cervical transformation zone. High infection of HPV in women is due to large area of immature squamous metaplastic cells, more commonly seen in younger women. In our study, there was strong expression of p16 (nuclear or cytoplasmic) in the squamous metaplastic cells, indicating an active HPV infection in these cells (63.6%).

The present study showed an overall positivity of 27.2% for Ki67. Kruse et al stated Ki 67 as a useful diagnostic adjunct to differentiate CIN grades. And also, as a sensitive indicator of progression of low grade CIN. This study, showed strong KI 67positivity in high grade lesion than in low grade lesion with a significant p value of 0.010.

P 63 in our study showed an overall positivity of 62.8%. P63 is linked to the maturation of normal squamous epithelium. In the study by Bradley et al, P63 expression was lost when the cell transformed from squamous to glandular differentiation and retained expression when cell changed from columnar to squamous. In the present study, the endocervical glandular cells showed a strong expression for P63 which indicates a possibility that this area may be undergoing squamous metaplastic change. P63 also showed the best association with the progression of the lesion (benign or malignant) with a sensitivity and specificity of 78% indicating that this may be a better marker to identify the progression of the lesion.

In the study by Charles J et al, p63 expression was purely confined to normal and benign cells and squamous cell carcinoma. A combination of P16 with ki67 is useful to determine the viral load (P16) and the degree of malignant change (Ki67) in the cell. P63 expression is linked to the change in differentiation in a cell, when the cell changes from a columnar type to a squamous type, thus may be used as a marker in identifying cells that undergo early squamous metaplasia. The combination of all the three helps to narrow down the exact focus of cells which are infected by HPV 16 (P 16ink4a), undergoing a squamous metaplasia (by p 63), and changing to a dysplastic or malignant cell (by Ki67).²³⁻²⁸

CONCLUSION

P16 identifies HPV 16 infection in uterine cervix, but it is not a sensitive marker to predict the progression of a lesion. Ki67 and P 63 are helpful in determining the nature of progression of a lesion whether the metaplasia is due to inflammation or atypical change. High expression of Ki 67 indicates a neoplastic progression. P63 may be used to differentiate benign from malignant lesions. P16 with P63 combination also showed good results in predicting the progression of a lesion. More elaborate studies are needed to confirm this finding.

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