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Analysis of Immunohistochemical Expression of $P^{16INK4a}$ in Preneoplastic Squamous Cell Lesions of Cervix

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Abstract
Aims*: The purpose of this study were to evaluate the results of expression of p16INK4a in preneoplastic squamous cell lesions of cervix in order to assess the association of HPV infection in those lesions and to study the pattern of expression of p16 and also to compare p16 expression in various histological types of cervical preneoplastic squamous cell lesions by immunohistochemistry.*
Methods: *Immunohistochemical analysis of p16 expression was performed on 34 paraffin embedded tissue samples, obtained from cervical biopsy including 25CIN I, 5CIN II, and 4CIN III by using commercially available mouse monoclonal antibody to p16 (clone G175 – 405). Two parameters were evaluated in p16 expression: Percentage of p16 positive cells and reaction intensity of p16 immunostaining. The p16 expression was graded as negative, Grade 1, 2, 3 and its reaction intensity was graded as negative, weak, moderate and strong.*
Results: *In the present study out of 34 cases, the incidence of CIN I constituted majority of the preneoplastic lesions of cervix (73.5%). p16 expression was seen in 28% of CIN I, 80% of CIN II and all CIN III cases. Only one CIN I case showed grade3 staining and strong reaction intensity, but most of the CIN II (60%) and CIN III (100%) cases showed grade 3 staining. In our study there was a statistically significant correlation between p16 expression, reaction intensity and lesion severity.*
Conclusion*: In the present study out of 34 preneoplastic lesions, totally 44.12% of cases showed p16 positivity. So p16 may be useful as an adjunct in histological sections to know the association of HPV in preneoplastic lesions to predict the risk of progression of the disease and to plan proper treatment. In this study p16 expression was correlated well with increasing grade of CIN. So p16 has significant implication in diagnostic, prognostic and preventive aspects of cervical cancer.*
Keywords: *Cervical intraepithelial neoplasia, Human papilloma virus, Immunohistochemistry, P16INK4a.*

I. Introduction

Cervical cancer is the fourth most common cancer among women worldwide, with an estimated incidence of 5,28,000 cases and 2,66,000 deaths in 2012 and it is most frequent among women between 15 to 44 years of age.[1]Current estimates indicate that incidence of 1,23,000 cases and 67,000 deaths due to cervical cancer occurred in India, contributing 23.2% and 25.2% to the global cervical cancer incidence and mortality respectively.[2] Invasive squamous cell carcinoma is preceded by precancerous changes in the cervical epithelium which are described previously as dysplasia and now as cervical intraepithelial neoplasia (CIN) . It has been firmly established that the Human papilloma virus infection plays an important role in cervical carcinogenesis.[3] Human papilloma virus are small, circular double stranded DNA viruses that belong to the papillomaviridae family. Experimental studies have identified nearly 200 types of human papilloma viruses, of those more than 40 have been identified in the genital tract and is classified into low risk and high risk categories based on the association with invasive cervical carcinoma.[4] HPV16, 18, 31, 33 and 45 are examples of high-risk types, while HPV6 and 11 belong to the low-risk types.[5] HPV – DNA consist of distinct three different regions. They are early region (ER), late region (LR) and upstream regulatory region (URR).The early region is composed of seven genes, E1 – E7, which play a significant role in viral replication and have oncogenic properties.

In normal cell cycle, the hypophosphorylated retinoblastoma (RB) in complex with E2F transcription factors prevents the progress of cell cycle from G1 to S. When RB is phosphorylated by cyclin D - cyclin-dependent kinase 4 (CDK4), it releases E2F. The latter then induces target genes resulting in progression of the cell cycle.
$P^{16INK4a}$ (henceforth referred to as p16) is a tumor suppressor protein normally binds to CDK4, inhibiting their association with cyclin D. The inhibition of the complex cyclin D - CDK4 prevents phosphorylation of RB leading to inhibition of cell cycle progression through G1- to S-phase.[6, 7]

The two viral oncoproteins in HPV namely E6 and E7 are mainly responsible for the progression of neoplasm. The E6 oncoprotein of high risk HPV causes degradation of p53, a tumor suppressor gene thus preventing cell cycle arrest or apoptosis. Similarly HPV E7 oncoprotein bind and inactivates the tumor suppressor protein pRB, resulting in release of the transcription factor E2F from its bound state allowing it to enter in to the nucleus. Once in the nucleus E2F promotes the transcription of target genes that are essential for cell cycle progression. As RB-E2F bound form normally inhibits transcription of the p16 gene, functional inactivation of pRB by the HPV E7 oncoprotein results in over expression of p16.[8 - 11]

The p16 protein is associated with cell cycle regulation and not with proliferation, its expression is not seen or is expressed in very low levels in normal cells and actively proliferating cells.P16 is strongly expressed in tumor cells affected by HPV and may be easily detected by IHC. Hence, over expression of it may serve as a surrogate biomarker of HPV infection which makes it useful in evaluating HPV associated preneoplastic lesions of cervix.[12-14]

Although there are several previous reports on the role of p16 in cervical cancer, Indian literature search revealed meager data exclusively correlating HPV and preneoplastic lesions of cervix, despite the fact that Indian females represent a major proportion of the affected population.

Hence, this study is an attempt to analyze the association of HPV infection by using p16 immunostaining in preneoplastic squamous cell lesions of cervix and evaluate its etiological and prognostic benefits as a valuable marker for cervical neoplasm.

II. Materials And Methods

This cross sectional study was a prospective study of a total of 34 cases including 25 CIN I cases, 5 CIN II cases and 4 CIN III cases, conducted in the department of pathology, Chengalpattu medical college, Chengalpattu during the period of June 2014 to August 2015. Ethical clearance for the study was obtained from the Institutional Ethics Committee of Chengalpattu Medical College, Chengalpattu. Tissue blocks of patients who were diagnosed as CIN (Cervical Intraepithelial neoplasia) I, II and III on histopathological examination as per standard protocol were included in this study. The overall age range of the 34 patients was 27 years to 83 years.

Materials used

Tissue sections from formalin fixed paraffin embedded tissues Haematoxylin and eosin stain
p16^{INK4a} monoclonal antibody kit (Mouse monoclonal, Clone (G175-405)

Immunohistochemistry for p16

Immunohistochemistry was performed on the 34 study sections. 4-micrometer thin sections were cut & placed on charged slides and incubated at 60 – 70 degree Celsius for 1 hour. Sections were deparaffinized in xylene for 15 minutes and rehydrated through graded alcohol by washing twice in absolute alcohol and in 90%, 70% alcohol for 5 minutes. Then sections were washed in distilled water two changes for 2 minutes each. Antigen retrieval was carried out at 150 degree Celsius in citrate buffer solution (pH = 9) for 15 min and washed in Tris Buffer Solution for 20 minutes. The slides were cooled to room temperature and washed in distilled water for 2 changes 5 minutes each and then washed in Tris Buffer Solution for 2 minutes. By adding 1% hydrogen peroxide on the sections and keeping them for 5 minutes the endogenous peroxidase activity was blocked. The slides were washed in buffer solution for 2 minutes each. Then, primary antibody (p^{16INK4a}– clone G175 – 405 – Mouse monoclonal antibody) was added and kept for 30 minutes at room temperature then washed in buffer solution twice, two minutes each. Secondary antibody (Polyexcel Target binder reagent) was applied and kept for 15 minutes then washed in two changes of buffer 2 minutes each, followed by incubation with Horse radish peroxidase for 15 minutes. Colour was developed by incubating the sections with diamino benzidene for 5 minutes then washed in distilled water and sections were counter stained with haematoxylin. The slides were washed in running tap water for 3 minutes. The slides were air dried, cleared with xylene and mounted with DPX. For positive control – Histological sections of Squamous Cell Carcinoma Cervix with known P16 positivity was included in each batch of staining. For negative control – Phosphate buffer solution was used instead of primary antibody.

Interpretation of staining results and statistical analysis

p16, immunostained sections were reviewed. Chestnut brown colour in the nucleus and/or cytoplasm was considered as immuno positivity. Two parameters were evaluated in p16 expression. (1)Percentage of p16 positive cells. (2)Reaction intensity of p16 immunostaining.

The percentage of p16 positivity was graded by counting the number of p16 immunoreactive cells in different epithelial clusters that is percentage of cells showing diffuse, strong brown nuclear and/ or cytoplasmic reactivity. It was graded as negative when no cells stained or cells showed only weak cytoplasmic staining. Grade 1, 2 and 3 were assigned based on the number of positive cells and graded 0–5%, >5–25% and >25%, respectively.[15] The intensity of the reaction was scored as negative, weak, moderate, and strong .[16]

Statistical Analysis

The data was analyzed by using SPSS statistical software version 16. Continuous data was expressed as mean and median. Correlation between histopathological results and immunohistochemistry results were calculated by chi-square test. P values less than 0.05 were regarded to be statistically significant.

III. Results

In the present study out of 34 cases, the incidence of CIN I constituted majority of the preneoplastic lesions of cervix (Table 1). From table 1, it is evident that, low grade preneoplastic lesions (CIN I) are seen most commonly in 41 – 50 years of age, high grade lesions (CIN II, III) are most commonly seen in 51 – 60 years of age. The age range of the 34 patients in this study was 27 years to 83 years with a median age 45 years. Mean age of CIN I was 42.40years, CIN II was 54.80 years and CIN III was 57.25 years.

Among CIN I group, majority of them (72%) were observed p16 negative in contrast to CIN II and CIN III, in which most of the cases showed p16 positivity. p16 expression was seen in 28% of CIN I, 80% of CIN II and all CIN III. On making comparison between p16 expression versus different grades of CIN, it was found to be statistically significant (P value = 0.001) (Table 2) (Figure1). Only one CIN I case showed grade3 staining and strong reaction intensity, but most of the CIN II (60%) and CIN III (100%) cases showed grade 3 staining (Table 2). In the present study it was noted that 28% of CIN I, majority of the CIN II and CIN III showed strong reaction intensity for p16 immunostaining (Table 3).

In this study there was a statistically significant correlation between p16 expression, reaction intensity and lesion severity (p value = 0.001). Moreover, expression of p16 is increased with increasing grades of CIN.

IV. Discussion

Cervical cancer is one of the leading causes of morbidity and mortality among women worldwide. Many studies revealed the association of human papilloma virus infection in both precancerous and invasive cervical cancer. Most of the HPV infection are transient, if it persists the risk of developing preneoplastic lesions increases as well as the risk of developing cervical cancer.[17] Pap smear screening and histopathological examination and interpretation of cervical biopsy specimen has markedly reduced the number of deaths due to cervical cancer, however they give little information regarding the association of HPV infection, risk of progression or regression and prognosis. Most of the low grade intraepithelial lesions and some of the high grade lesions regress spontaneously overtime without treatment.[18] So overtreatment of patients who will not benefit from treatment or under treatment of patients who have the risk of progression, underscores the importance of detection of HPV in those lesions.

P16 has recently emerged as a surrogate marker of HPV, used both in cytology and histology sections, it significantly reduces the interobserver variability when diagnosing cervical intraepithelial lesion, furthermore it's over expression appears to correlate with degree of cervical intraepithelial neoplasia.[19]

P16 over expression in low grade cervical intraepithelial lesion have a higher risk of persistence and progression to high grade lesion.

In future we can plan HPV vaccine trials in areas with HPV high prevalence. So identifying the association of HPV in preneoplastic lesions has significant implication in diagnostic, follow-up, prognostic and preventive aspects of cervical cancer.

The lesion with highest incidence was CIN I in this study and is similar to the incidence quoted by Wang et al, Tan et al and Nam et al.[20,21,22] In the present study, p16 over expression was seen only in 28% (7/25) of CIN I cases. Sano T et al indicated that low risk HPV E7 oncoprotein have no effect on p16 expression, because its affinity is 10 times lower than that high risk HPV E7 oncoprotein.[9] Tan et al in 2010 stated that low grade lesion with p16 negativity may be due to infection with low risk HPV or due to subclinical infection.[21] In this study, p16 over expression was seen in 80% (4/5) of CIN II and 100%(4/4) of CIN III cases. Our findings are similar to Benovolo et al (2006), who observed over expression of p16INK4a in 31% (17/54) of CIN I, 90% (9/10) of CIN II and all cases (11/11) of CIN III. [23]The results of expression of p16 in all grades of CIN was statistically significant (p value = 0.001) and is concordant with the most of the literatures.[15, 21,23,24,25]

A study by volgareva et al in 2004, observed that some of the preneoplastic and neoplastic lesions of cervix do not express p16. They suggested that due to lack of p16 positivity we should not exclude a patient from risk group. They concluded that absence of p16 expression may be due to p16 mutation, deletion or hypermethylation.[25] In the present study it was noted that there was a significant increase in the grade of p16 expression, when we moved from low grade cervical intraepithelial lesion to squamous cell carcinoma and the correlation was found to be statistically significant (p value = 0.001).

Ishikawa et al analyzed the p16 expression and HPV typing in cervical lesions and found that p16 over expression in CIN I was more common in patients with HPV 16 and 52.[26]In the present series one CIN I case showed grade 3 expression of p16 which need greater attention, because of increased risk of persistence and progression to high grade lesion. So p16 could be used as a suitable marker of HPV infection to predict the outcome of CIN lesions. Kleas et al in 2001 correlated the p16 expression in cervical lesions to the HPV status of the sample by using PCR. They graded the p16 expression as negative, sporadic, focal and diffuse. All CIN I lesions with low risk HPV showed no diffuse staining, but CIN I with high risk HPV displayed diffuse p16 expression. Finally they concluded that p16 may be useful to identify the dysplastic cervical cells in both cytology and histopathology.[15]

Izadi mood et al in 2012 observed a direct relationship between reaction intensity and lesion severity. They concluded that p16 reaction intensity was superior to any other analyzed parameter and they found it being the best indicator of p16 expression.[16] In the present study majority of the low grade CIN showed negative reaction intensity for p16. Most of the high grade CIN cases in this study showed strong reaction intensity, while only one patient among those with high grade CIN failed to express this marker. There was a statistically significant relationship between histopathological diagnosis and p16 reaction intensity in our study (p value = 0.001) and our findings are concordant with the above literature. The limitation of our study was we did not attempt for HPV DNA detection studies to validate the utility of p16 for detection of HPV in cervical neoplasm.

V. Conclusion

Following conclusions were arrived from this study:-

- As nearly 89% of high grade lesions of cervix (CIN II, CIN III) and 28% low grade lesions (CIN I) showed p16 over expression in our region, further studies can be undertaken to evaluate the prevalence of high risk HPV in general population, which can help us to take necessary steps to minimize and prevent the infection through health education and HPV vaccination.
- Low grade cervical intraepithelial lesions showed less reaction intensity and less grade of staining. High grade cervical intraepithelial lesions cases showed strong reaction intensity and higher grade of staining. P16 expression was progressively increased with increasing grades of cervical neoplasm. So p16 may be useful as an adjunct in histological sections to detect HPV in those lesions which can help us to predict the progression of disease.

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Table 1: Age wise distribution of cervical squamous lesions

AGE GROUP	CIN I n = 25 (73.5%)	CIN II n = 5 (14.7%)	CIN III n = 4 (11.8%)
< 30 Years	5 (20%)	0	0
31-40	7 (28%)	1 (20%)	1 (25%)
41-50	8 (32%)	1 (20%)	1 (25%)
51-60	3 (12%)	2 (40%)	1 (25%)
>60	2 (8%)	1 (20%)	1 (25%)

Table 2: Grading of P16 ^{Ink4a} expression in preneoplastic squamous cell lesions of cervix

Histological diagnosis	Grading of p16 INK4A staining			
	P16 negative	Grade 1	Grade 2	Grade 3
CIN I (n=25)	18/25 (72)%	3/25 (12%)	3/25 (12%)	1/25 (4%)
CIN II (n=5)	1/5 (20%)	0/5 (0%)	1/5 (20%)	3/5 (60%)
CINIII (n=4)	0/4 (0%)	0/4 (0%)	0/4 (0%)	4/4 (100%)
Total	19/34 (55.88%)	3/34 (8.82%)	4/34 (11.77%)	8/34 (23.53%)

Chi sq = 23.2, p value = 0.001

Figure 1: Grading of p16 ink4a expression in preneoplastic squamous cell lesions of cervix

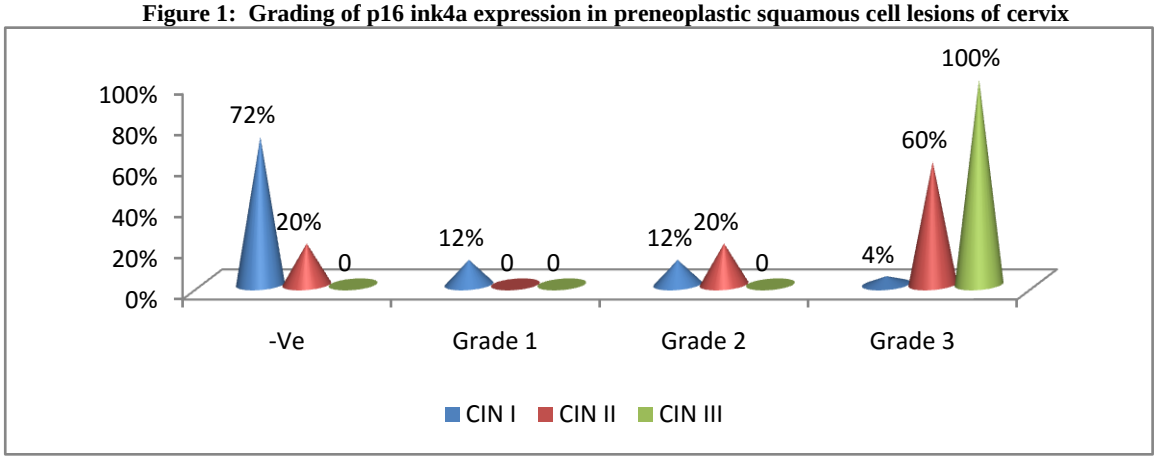

Table 3: Correlation between histopathological diagnosis and reaction intensity of p16 staining

Histological diagnosis	Reaction intensity for p16INK4A			
	Negative	Weak	Moderate	Strong
CIN I	18/25 (72%)	0/25 (0%)	6/25 (24%)	1/25 (4%)
CIN II	1/5 (20%)	0/5 (0%)	1/5 (20%)	3/5 (60%)
CIN III	0/4 (0%)	0/4 (0%)	0/4 (0%)	4/4 (100%)
Total	19/34 (55.88%)	0/34 (0%)	7/34 (20.59%)	8/34 (23.53%)

Chi sq = 22.33, p = 0.001

Table 4: Comparison of P16 Positivity in CIN with Other Studies

Sl. no.	Authors	CIN1	CIN2	CIN3
1.	Klaes et al 2002	15/17 (88%)	10/10 (100%)	43/43 (100%)
2.	Benovolo 2006	17/54 (31%)	9/10 (90%)	11/11 (100%)
3.	Kim et al 2015	22/31 (70.9%)	21/25 (84%)	41/41 (100%)
4.	Tan et al 2010	16/60 (26.7%)	9/21 (42.9%)	46/48 (95.9%)
5.	Present Study	7/25 (28%)	4/5 (80%)	4/4 (100%)

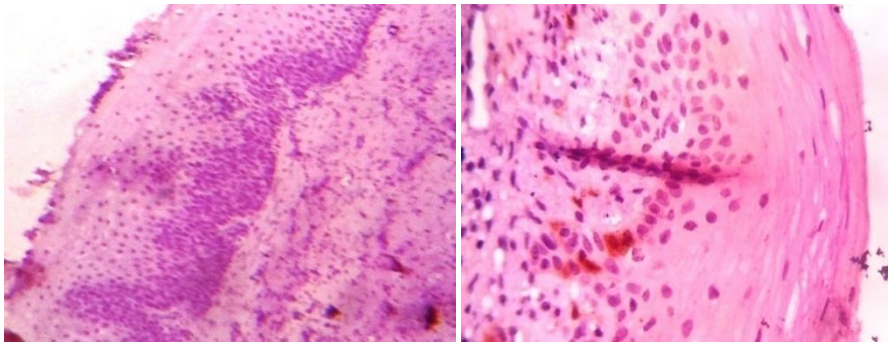

Figure 2 &3: p16 IHC staining in Cervical intraepithelial neoplasia I (CIN I) showing negative, grade1 immunostaining and moderate reaction intensity in figure 3. (100X)

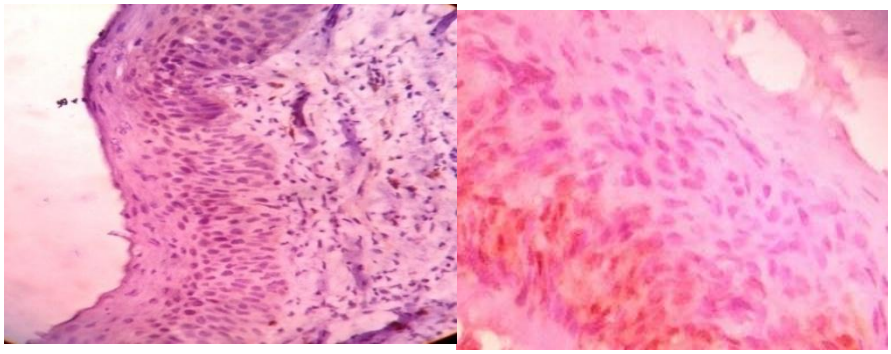

Figure 4 & 5: p16 IHC staining in Cervical intraepithelial neoplasia II (CIN II) showing grade 2, grade 3 immunostaining, moderate reaction intensity in figure 4 and strong reaction intensity in figure 5 . (400X)

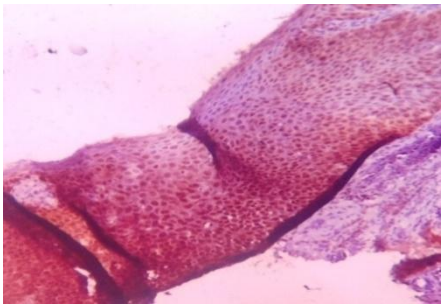

Figure 6: p16 IHC staining in cervical intraepithelial neoplasia III (CIN III) showing grade 3 immunostaining and strong reaction intensity. (400X)

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4.Materials And Methods	Weak , moderate ,	weak, moderate,
5.Results	cervix(Table 1).	cervix (Table 1).
5.Results	II(60%)	II (60%)
5.Results	CIN III(100%)	CIN III (100%)
5.Results	staining(Table 2	Staining (Table 2
5.Discussion	expression .	expression.