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ANALYSIS OF IMMUNOHISTOCHEMICAL EXPRESSION OF $P^{16INK4a}$ IN NEOPLASTIC SQUAMOUS CELL LESIONS OF CERVIX

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ABSTRACT

Introduction: ***Cervical cancer is caused by persistent infection by high risk Human papilloma virus (HPV).Increasing expression of HPV viral oncogenes might be reflected by increased expression of p16. Hence immunohistochemical detection of $p16^{INK4a}$ is an easy and cost effective method than molecular detection of HPV.***

Aims: ***The purpose of this study were to evaluate the results of expression of $p16^{INK4a}$ in neoplastic 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 neoplastic squamous cell lesions by immunohistochemistry.***

Methods: ***Immunohistochemical analysis of p16 expression was performed on 26 paraffin embedded tissue samples, obtained from cervical biopsy including 2 early invasive squamous cell carcinoma (SCC), 6 large cell keratinizing SCC, 16 large cell non- keratinizing SCC and 2 cases small cell non-keratinizing SCC 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 26 cases, the incidence of large cell non- keratinizing SCC constituted majority of the neoplastic lesions of cervix (61.5%). Most of the SCC (96.15%) showed grade 3 scoring for p16 positivity except one case which showed grade 2 scoring. Majority of SCC cases (96%) showed strong reaction intensity for p16 immunostaining.***

Conclusion: ***In this study of 26 neoplastic sqaumous cell lesions, all patterns of cervical neoplasia showed p16 positivity. P16 may be useful as an adjunct in histological sections to detect HPV in those lesions.***

Keywords: - Squamous cell carcinoma, Human papilloma virus, Immunohistochemistry, $P16^{INK4a}$.

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 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 is 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 hypophos-phorylated 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 CDK 4, 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 RB, 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 neoplastic 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 neoplastic 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 neoplastic squamous cell lesions of cervix and evaluate its etiological and prognostic benefits as a valuable marker for cervical neoplasm.

MATERIALS AND METHODS

This cross sectional study was a prospective study of a total of 26 (n=26) including 2 early invasive squamous cell carcinoma (SCC), 6 large cell keratinizing SCC, 16 large cell non-keratinizing SCC and 2 small cell non-keratinizing SCC 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 various histological types of squamous cell carcinoma on histopathological examination as per standard protocol were included in this study. Tissue blocks of squamous cell carcinoma patients who underwent radiotherapy or chemotherapy were excluded from this study. The overall age range of the 26 cases was 35 to 73 yrs.

Materials used

Tissue sections from formalin fixed paraffin embedded tissues Haematoxylin and eosin stain $p16^{INK4a}$ monoclonal antibody kit (Mouse monoclonal, Clone (G175-405) **Immunohisto-chemistry for p16** Immunohistochemistry was performed on all the 26 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 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. Color 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 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.

RESULTS

In this study out of 26 cases of squamous cell carcinoma, the incidence of large Cell non-keratinizing SCC (61.5%) constituted majority of the neoplastic lesions of cervix (Table/Figure 1). The age range of the 26 patients in this study was 35 years to 73 years with a median age of 58 years. Mean age of SCC was 54.92 years.

(Table/Figure 1): Distribution of Various Histological Subtypes of Squamous Cell Carcinoma of Cervix

NATURE OF LESION IN CERVIX	DISTRIBUTION	
	NUMBER OF CASES n = 26	PERCENTAGE
Early Invasive SCC	2	7.7%
Large Cell Keratinizing SCC	6	23.1%
Large Cell Non- Keratinizing SCC	16	61.5%
Small Cell Non-Keratinizing SCC	2	7.7%

All patterns of cervical neoplasia including 2 early invasive squamous cell carcinoma (SCC), 6 large cell keratinizing SCC, 16 large cell non-keratinizing SCC and 2 cases small cell non-keratinizing SCC showed p16 positivity. Most of the SCC cases (96.15%) showed grade 3 scoring for p16 positivity except one case which showed grade 2 staining. (Table/Figure 2). On making comparison between p16 expression grading versus different histological types of squamous cell carcinoma , it was found to be statistically not significant (P value = 0.3) .

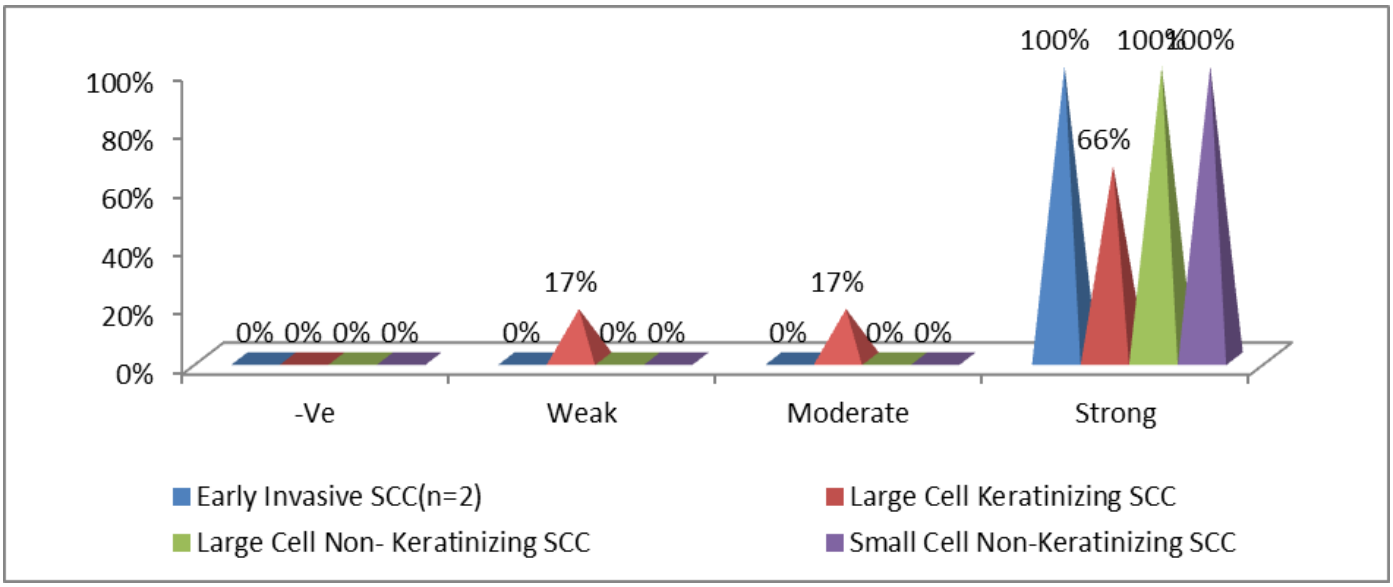
(Table/Figure 2): Grading of P16 Ink4a Expression in Neoplastic Squamous Cell Lesions of Cervix

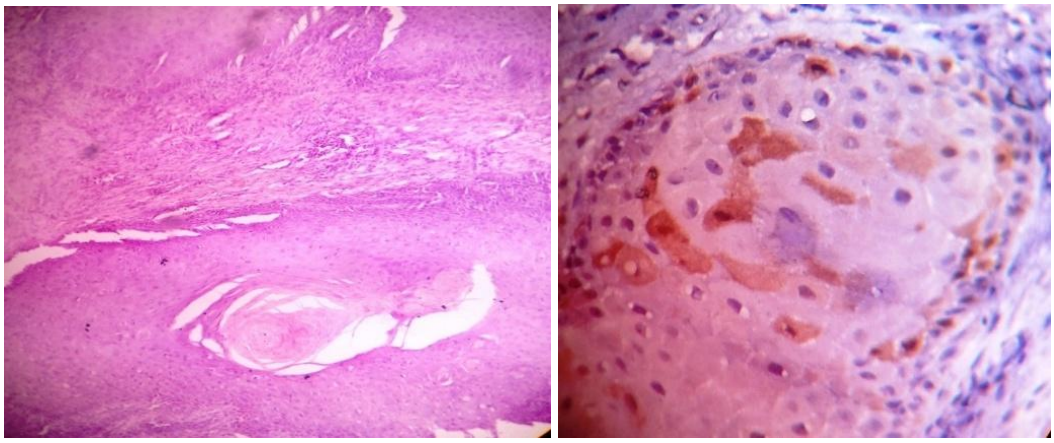
LESION	Negative	Grade 1	Grade 2	Grade 3
Early Invasive SCC (n=2)	0/2(0%)	0/2(0%)	0/2(0%)	2/2(100%)
Large Cell Keratinizing SCC (n=6)	0/6(0%)	0/6(0%)	1/6 (17%)	5/6 (83%)
Large Cell Non- Keratinizing SCC (n=16)	0/16(0%)	0/16(0%)	0/16(0%)	16/16 (100%)
Small Cell Non-Keratinizing SCC (n=2)	0/2(0%)	0/2(0%)	0/2(0%)	2/2 (100%)
Total	0/26(0%)	0/26(0%)	1/26(3.85%)	25/26(96.15%)

In this study it was noted that 24 cases (96%) of SCC showed strong reaction intensity for p16 immunostaining, whereas one case showed weak reaction intensity and one case showed moderate reaction intensity for p16 immunostaining. (Table/Figure 3 and 4). But the correlation between histological diagnosis, p16 expression and reaction intensity was found to be statistically not significant (P value = 0.3).

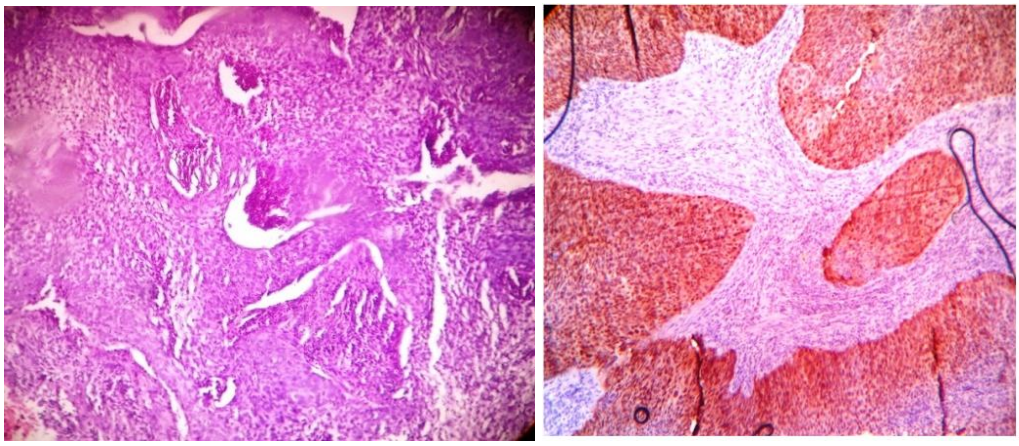
(Table/Figure 3): Correlation between histopathological diagnosis and reaction intensity of p16 staining:

LESION	Negative	Weak	Moderate	Strong
Early Invasive SCC(n=2)	0/2(0%)	0/2(0%)	0/2(0%)	2/2 (100%)
Large Cell Keratinizing SCC (n=6)	0/6(0%)	1/6 (17%)	1/6 (17%)	4/6 (66%)
Large Cell Non- Keratinizing SCC (n=16)	0/16(0%)	0/16(0%)	0/16(0%)	16/16 (100%)
Small Cell Non-Keratinizing SCC (n=2)	0/2(0%)	0/2(0%)	0/2(0%)	2/2 (100%)
Total	0/26(0%)	1/26(3.85%)	1/26(3.85%)	24/26(92.30%)

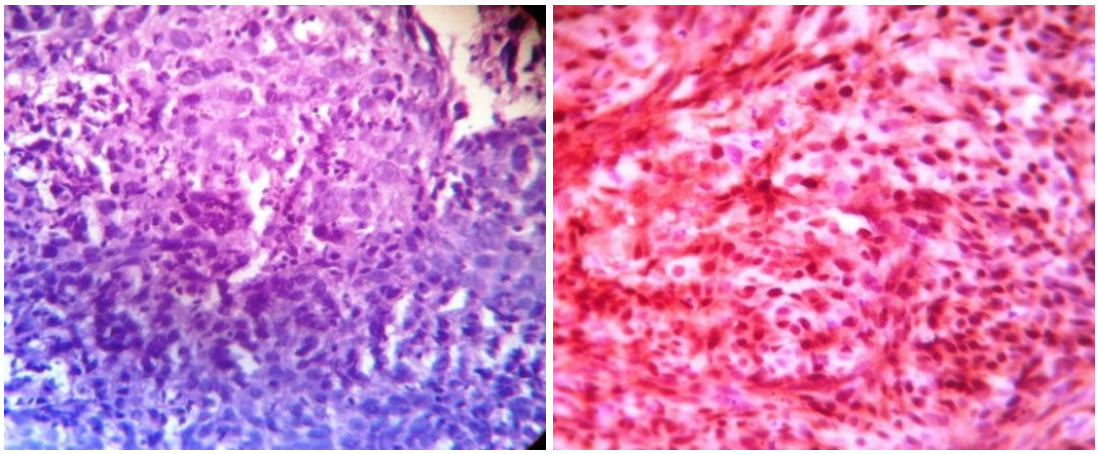




(Table/Figure 5): Large cell keratinizing squamous cell carcinoma H&E(100X).
(Table/Figure 6): P16 immunostaining shows grade2 immunostaining and moderate reaction intensity (400X).



(Table/Figure 7): Large cell non keratinizing squamous cell carcinoma H&E (100X).
(Table/Figure 8): P16 immunostaining shows grade 3 immunostaining and strong reaction intensity.



(Table/Figure 9): Small cell non keratinizing squamous cell carcinoma H&E (100X).
(Table/Figure 10): P16 immunostaining shows grade 3 immunostaining and strong reaction intensity.

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 infections 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. Furthermore absence of detection of HPV in cervical cancer cells is associated with poor prognosis. [18] Hence HPV detection in those lesions play a pivitol role to differentiate HPV positive from HPV negative SCC by which we can assess the prognosis of the patient.

So identifying the association of HPV in neoplastic lesions has significant implication in diagnostic, prognostic and preventive aspects of cervical cancer.

In this study majority of them were of large cell non keratinizing subtype (61.5%) (Table/Figure 1). The incidence of this is similar to the incidence quoted by Adisorn Jedpiyawongse et al.[19] Non keratinizing tumors

of cervix had better prognosis when radiotherapy is used, but there was no significant difference in the prognosis when the treatment is surgical.

p16 positivity was noted in all 26 cases of squamous cell carcinoma in this study. Our findings are similar to those of supriya srivatsava, Adisorn Jedpiyawongse et al, Benovolo et al and Kim et al. [8, 19-21] 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. [22]

Most of the SCC (96.15%) showed grade 3 scoring for p16 positivity except one case which showed grade 2 scoring. Our study is broadly in accordance with results of the previous publications. [8, 10, 13, 14]

A study by Supriya srivastava (2010) analyzed the expression of both p16 and MIB1 in cervical lesions and normal cervical epithelium. They found the expression of both in all cases of CIN I, II, III and cancer cervix except in normal cervical epithelium. In their study they grade the number of p16 positive cells as grade 0(0% positive cells), grade 1(1 – 10% positive cells), grade 2(10 – 50% positive cells) and grade 3 (>50%p16 positive cells). [8]

A study by Riou G et al (1990) examined 106 cases of early invasive squamous cell carcinoma of cervix with HPV sequencing by PCR and southern hybridization and concluded that there was 2.6 times higher chance of relapse and 4.5 times higher chance of distant metastasis in HPV negative patients when compared to HPV positive patients. So detection of HPV in those lesions indicate better prognosis. [18]

Izadi mood et al in 2012 concluded that p16 reaction intensity was superior than any other analyzed parameter and they found it being the best indicator of p16 expression.[16] In our study, out of 26 SCC cases, 24 (96.15%) cases showed strong reaction intensity, one case showed moderate intensity and one case showed weak intensity. These 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.

CONCLUSION

In this study, large cell non keratinizing SCC was the commonest subtype of SCC comprising 61.5%. P16 expression was seen in all cases of squamous cell carcinoma (26/26) of tissue biopsies. Similarly most of the SCC (96.15%) showed grade 3 scoring and strong reaction intensity for p16 positivity.

p16 expression is a valuable marker of HPV. Moreover, HPV positive cervical carcinoma indicate better prognosis than HPV negative ones, analysis of p16 expression may be useful as one of a prognostic indicator, so it should be incorporated into routine surgical pathology practice.

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