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Assesment and clinicopathological correlation of P16 expression in oral squamous cell carcinoma

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Abstract
Introduction: Oral squamous cell carcinoma is a major cause of death throughout the developed world. It is associated with smoking and alcohol consumption. Human papillomavirus (HPV) type 16 has also been suggested to play a role in etiology of head and neck squamous cell carcinoma. p16 expression is now being used as a surrogate marker of HPV infection in squamous cell carcinoma and provides important prognostic information and future therapy planning.
Materials and Methods: In this observational cross-sectional study, total 60 cases of oral squamous cell carcinoma were taken. Tumor grade was determined according to WHO criteria. p16 expression was determined by immunohistochemical staining. The obtained results were analyzed and evaluated using Chi-square test, P value of < 0.05 was taken significant.
Results: Out of 60 cases, 39 cases are positive for P16 out of which 19 cases show strong positivity.
Conclusion: As HPV integration with transcription of viral oncoprotein induces overexpression of p16, immunohistochemical expression of p16 can be used as a surrogate marker of HPV. This approach can be implemented in diagnostic laboratories and can provide support for vaccination program in high risk group.

Keywords: Oral squamous cell carcinoma, human papillomavirus, immunohistochemistry, p16

Introduction

Oral squamous cell carcinomas represent a major health issue, with over 2 lac new cases reported worldwide annually. Commonly there is history of tobacco exposure and alcohol abuse. Though improvements in screening and early diagnosis have dramatically reduced the incidence of these neoplasms in recent years, the 5 years disease free survival is still poor. Recently several studies have shown that HPV are clearly involved in the pathogenesis of a subgroup of oral squamous cell carcinoma [1, 2].

This distinct subgroup of carcinoma is characterized by distinctive prognostic features: HPV infection, better response to induction chemotherapy and concurrent chemo-radiation protocol and an overall better clinical outcome, as compared to HPV negative oral squamous cell carcinoma [3, 4].

Several studies using a variety of techniques, including immunohistochemistry, in situ hybridization, dot blot and southern blot hybridization, and polymerase chain reaction (PCR), have since been able to demonstrate the presence of HPV genome in the cells of some cases of Head and neck squamous cell carcinoma [5].

p16 expression now being used as a surrogate marker of HPV infection in oral squamous cell carcinoma [6, 7, 8, 9, 10]. This may help in providing important prognostic information and future therapies aimed at targeting this pathway of HPV tumorigenesis.

P16 gene functions as a negative regulator of cell cycle progression through its inhibition of cdk4/6 which in turn determines the blockage of cyclin dependent phosphorylation of retinoblastoma protein (Rb). Mechanism of inactivation include homozygous gene deletion, gene mutation and hypermethylation of upstream CpG island regions [11].

HPV-associated cancers are caused by expression of HPV's E6 and E7 proteins that bind to and inactivate tumor suppressor proteins p53 and retinoblastoma protein (pRb), respectively leading to malignant transformation of HPV infected cells [13] pRb is functionally inactivated by binding of viral protein and no longer acts as a cell cycle inhibitor. HPV E7 binds to hypophosphorylated form of Rb. The binding occurs in Rb pocket that sequesters E2F transcription factors.

Thus pRb, unable to bind the E2F transcription factors, is functionally inactivated and transcription factors are free to cause cell cycle progression [12].

Objectives

1) To evaluate p16 expression in oral squamous cell carcinomas.
2) To study other associated risk factors.

Materials and Methods:

- Observational cross sectional type of study.
- **Total number of cases** - 60 cases of oral squamous cell carcinoma pertaining to oral cavity were studied.
- **Exclusion criteria** - Patients with other than oral squamous cell carcinoma such as adenocarcinoma, melanoma, sarcoma, metastasis, etc., were excluded.
- **Clinicopathological parameters** - age of patient, history of tobacco use, pan chewing,smoking, alcohol abuse, other relevant irritants, involvement of abnormal sexual habits, site of lesion, grade of tumour, and lymph node metastasis.
- Out Of 60 received samples 27 are whole specimens and 33 are biopsies.
- Hematoxylin and Eosin stain is done first and diagnosis is confirmed on microscopy for squamous cell carcinoma. Confirmed cases are selected for p16 immunohistochemistry and others are excluded.

Immunohistochemical analysis

Immunohistochemical (IHC) profile of the tumour was assessed by subjecting one representative section of tumour block to p16. Immunohistochemistry was performed on 4µm thick sections from 10% formalin fixed paraffin embedded specimens, according to the streptoavidin-biotin immunoperoxidase technique. Positive and negative control were run simultaneously. Strong brown immunoreactivity was considered as positive staining.

Interpretation of Results:

- The IHC expression of p16 was classified according to nuclear and cytoplasmic positivity.
- The biopsies were scored as positive when more than 5% cells (cut-off) stain positive and was graded as :-

1. Negative (0–5% of nuclei and cytoplasm positive),
2. Sporadic (5–10% of nuclei and cytoplasm with weak and scattered positivity),
3. Focal (>10–30% of labeled nuclei and cytoplasm strongly positive, spreading in one tissue area), and
4. Diffuse (>30–85% of labeled cells with strong positivity, spreading in several tissue areas).

Biopsies with diffuse pattern were considered to have high IHC expression of p16 (Grade III). Focal distribution was considered as moderate expression (Grade II) and sporadic positivity as low expression (Grade I). (06)

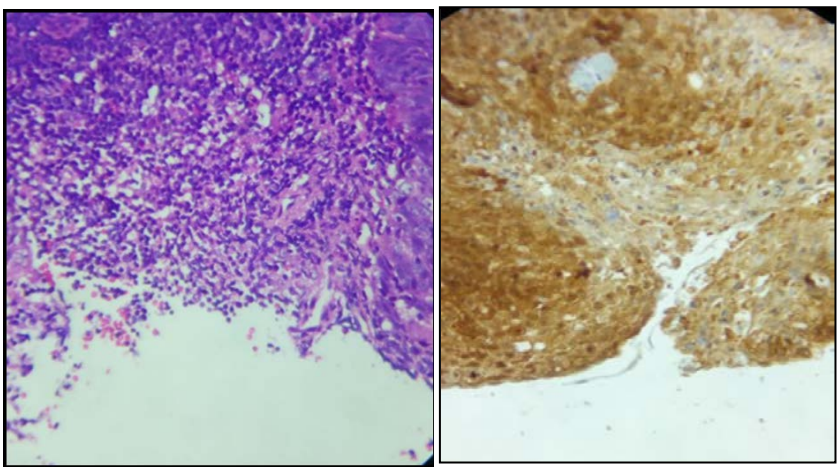

Fig 1: Diffuse p16 positivity

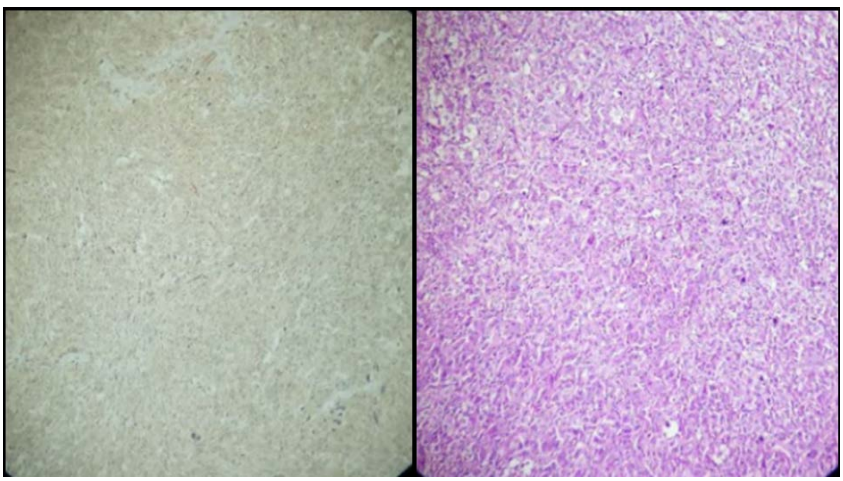

Fig 2: Weak p16 positivity

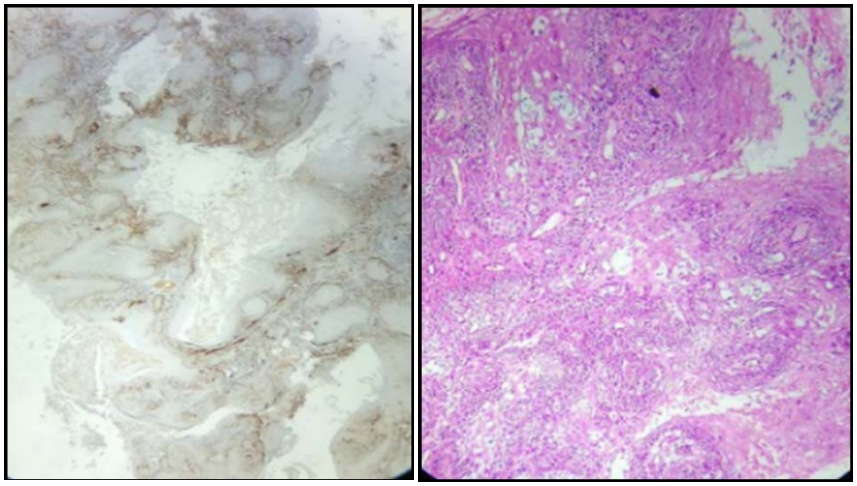

Fig 3: Moderate p16 positivity

Statistical analysis

A descriptive observational study was carried out for all the variables included in the study. The whole data was entered in Microsoft excel sheet and analysed EPI Info- 7 software. As the data was qualitative, chi-square test was used to assess the association between these parameters. A value of $p<0.05$ was taken as significant and <0.01 as highly significant; whereas, $p>0.05$ was taken as non-significant.

Observations

The age of the patients ranged from 27-75 years with a mean of 55.3 ± 12.2 years. It was observed that maximum cases were in the age group of >50years. The youngest patient was 27 years old and oldest was 75 years of age. Out of 60 cases 46 are males and 14 are females.

Out of 60 cases, 42 of the patients were history of tobacco chewing, 19 of the patients had history of smoking, 14 of the patient had history of alcohol consumption. 15 cases had history of pan chewing and 7 cases has positive history of multiple sexual partner.6 cases had positive for HIV and 7 cases had history lymph node metastasis. Tongue was the most common site for occurrence of oral squamous cell carcinoma in present study. WHO histological grading was done for all cases of oral squamous cell carcinoma. Majority of the cases 32 were Grade II followed by 8 cases of Grade III. Grade I tumour has 20 cases.

Table 1: Characteristic of Patients

Total Patient	60
Male Patient	46
Female Patient	14
Age Group Of Patient	27-75 year
Mean Age of Patient	55.3 years ±12.2 years

P16 expression

It was observed that 39(65%) cases were positive for p16 (inclusive of all grades), while 21(35%) cases were negative.

Out of 65% positive cases, 9 cases shows Grade I (low expression), 11 cases shows Grade II (moderate expression), 19 cases shows Grade III (strong expression).

Table 2 - illustrates correlation of p16 expression with various clinical parameters. Out of 19 cases with Grade III expression of p16, 11 cases belonged to age more than 50 years and 16 cases were males. No significant association was seen between p16 expression and age (p=0.96) and sex(p= 0.14).

Table 2: Correlation of P16 expression with age and sex of the patient

Clinical Parameters	0-5% P16 Negativ	5-10% P16 Sporadic	>10-30% P16 Focal	>30-85% P16 Diffuse	Total Cases	P value
	Grade 0	Grade I	Grade II	Grade III	n=60	
Age						
≤50 years	9	3	3	8	23	0.96
>50 years	12	6	8	11	37	
Total	21	9	11	19	60	
Sex						
Female	8	1	2	3	14	0.14
Male	13	8	9	16	46	
Total	21	9	11	19	60	

Table 3 illustrates correlation of p16 expression with various risk factors. Though majority of our patients are tobacco chewers, smokers and history of alcohol consumption, p16 expression was not significantly associated with these risk factors.

History of pan chewing was present in 15 cases in our study. However, p16 expression was significantly seen in these cases (p=0.01). A significant correlation of p16 expression was also seen in cases with multiple sexual partners (p=0.009).Significant correlation was also observed between p16 and tobacco chewing(p=0.01).There is no statistical correlation between smoking and alcoholism.

Table 3: Correlation of P16 Expression with Various Risk Factors

Risk Factors	0-5% P16 Negative	5-10% P16 Sporadic	>10-30% P16 Focal	>30-85% P16 Diffuse	Total cases	P value
	Grade 0	Grade I	Grade II	Grade III		
Smoking						
Negative	18	4	6	13	41	0.31
Positive	3	5	5	6	19	
Total	21	9	11	19	60	
Alcohol						0.81
Negative	18	5	8	15	46	
Positive	3	4	3	4	14	
Total	21	9	11	19	60	
Pan chewing						
Negative	20	6	8	11	45	0.01
Positive	1	3	3	8	15	
Total	21	9	11	19	60	
Multiple sexual partners						
Negative	21	8	11	13	53	0.009
Positive	0	1	0	6	7	
Total	21	9	11	19	60	
Tobacco						
Negative	3	2	4	19	18	0.01
Positive	18	7	7	10	42	
Total	21	9	11	19	60	

Table 4- illustrates correlation of p16 expression with histological grades of oral squamous cell carcinoma. Out of 60 cases, 20 cases are well differentiated, 32 cases are moderately differentiated and 8 cases are poorly differentiated. Significant correlation is not observed between p16 and grading of tumours (p=0.08)

Table 4: Correlation of P16 Expression with Histological Grading

Who Histo Grading	0-5% P16 Negativ	5-10% P16 Sporadic	>10-30% P16 Focal	>30-85% P16 Diffuse	Total	P value
	Grade 0	Grade I	Grade II	Grade III		
Grade I	7	6	6	1	20	
Grade II	11	1	5	15	32	0.08
Grade III	3	2	0	3	08	
Total	21	09	11	19		

Table 5: illustrates correlation of p16 expression with lymph node metastasis. Out of 60 cases, 53 cases are node negative at the time of presentation and 7 cases are node positive and out 7 cases 2 cases show Grade III positivity. There is no statistical correlation between lymph node positive and p16 expression (p=0.31)

Table 5: Correlation of P16 Expression with Lymph Node Metastasis

Lymph Node Mets	0-5% P16 Negative	5-10% P16 Sporadic	>10-30% P16 Focal	>30-85% P16 Diffuse	Total	P value
	Grade 0	Grade I	Grade II	Grade III		
Absent	18	8	10	17	53	
Present	03	01	01	02	07	0.31
Total	21	0 9	11	19	60	

Table 6 - illustrates: HIV positive cases are 6 out of 60 cases and 5 cases shows Grade III positivity.it is significantly correlated with p16 expression (p=0.02)

Table 6: Correlation of P16 Expression with HIV Positivity

Hiv Positive	0-5% P16 Negativ	5-10% P16 Sporadic	>10-30% P16 Focal	>30-85% P16 Diffuse	Total	P value
	Grade 0	Grade I	Grade II	Grade III		
Absent	21	8	11	14	54	
Present	0	01	00	05	06	0.02
Total	21	09	11	19	60	

Discussion

In the present study, Grade III p16 expression was observed in 19 cases, Grade II p16 expression in 11 cases and Grade I in 9 cases: whereas, 21 cases were negative for p16 and scored Grade 0. No significant association was seen between p16 expression and age (p=0.96) as well as sex distribution (p=0.14).

Majority of the cases included in the present study are tobacco chewers and smokers, alcohol consumers and non-pan chewers. The 60 cases of oral squamous cell carcinoma

analysed for expression of p16 showed no statistically significant association between p16 expression and smoking (p=0.31) and alcohol consumption (p=0.81).

However, statistically association between p16 expression and patients with history of pan chewing was observed (p=0.01). In our study p16 expression shows significant association between tobacco use (p=0.01). Similar to our study, *Smith et al.*, found a statistically association between p16 expression and tobacco use [7]. In contrast *Lazarus et al.*, found no statistically association between p16 expression and tobacco use [15].

The variability and difference in expression in our study as compared to other studies may be due to geographical distribution and difference in prevalence of various risk factors.

Many studies on head and neck squamous cell carcinoma have utilized p16 as a surrogate marker for HPV DNA. According to a study conducted by *Herrero et al.*, in 2003, HPV DNA was detected more frequently in oral cancer biopsies of patient who reported history of more than one sexual partner [16]. Keeping in view the above studies, history of multiple sexual partners and practice of abnormal sexual activity was taken in our study. In present study history of abnormal sexual habits was obtained in 0 cases and history of multiple sexual partners in 7 cases.

A significant association is seen between p16 expression and history of multiple sexual partners (p=0.009) highly significant. According to studies conducted *by Fregonesi et al., Smith et al., Klussmann et al.,Konig et al.,Singhi et al and pannone et al.*, p16 expression was strongly associated with HPV infected head and neck squamous cell carcinomas [6, 7, 8, 9, 10, 14].

No history of abnormal sexual habits was observed. This can be attributed to social and cultural difference and small number of patient providing the history of such practices as extracting such history from the patients in our society was not very easy as it is considered a social taboo.

Histological grade is a means of quantitating the degree of differentiation by applying a set of histological criteria. Well differentiated tumours are of low grade and poorly differentiated tumours are of high grade. It is an important factor associated with distant metastasis. It adds important information to clinical and pathological staging, helps to identify patients at high risk for distant metastasis for whom an efficient systemic treatment is mandatory [17]. In our study, maximum number of cases belonged to Grade II [32], Grade I and Grade III are 20 and 8 respectively.

In present study p16 expression has not statistically significant correlation with histological grading of tumour (P=0.77). Our findings are same with the study by *Yuen et al., and Dragomir et al* in which there is no significant association between p16 expression and tumour grade [18, 19]. In contrast study of *Muirhead et al* observed that p16 over expression was more likely to be detected with higher grade [20].

Lymph node involvement being an important prognostic variable. In the present study, lymph node, lymph node metastasis is seen in 7 cases and 53 cases were negative. There is no statistical association between lymph node metastasis and p16 positivity (p=0.31). *Muirhead et al., and Yuen et al.*, did not observe any statistically significant association between p16 expression and lymph node metastasis and concluded that expression of p16 significantly contribute to cell proliferation and tumour size, but it has no prognostic significance for nodal metastasis and survival [18, 20].

HIV positive cases in present study is 6 out of 60 cases Grade III positivity is seen in 5 cases and history of HIV positive is statistically significant with p16 (p=0.02). HIV infected individuals had increased p16 levels within the effector memory subset, indicating a possible role for this marker in impaired clonal expansion during antiviral effector function. Taken together chronic HIV infection is associated with elevated expression of cellular aging marker p16 in T cells. ART restored normal p16 levels in in the CD4+ T cell compartment, indicating that use of therapy can be of fundamental importance to normal cell cycling and maintaining immune homeostasis [21].

Conclusion

P16 immunohistochemistry is advocated as a surrogate marker of HPV infection. This distinct subgroup of oral squamous cell carcinoma with high p16 expression is characterized by distinctive prognostic features: HPV infection, better response to induction chemotherapy and concurrent chemo-radiation protocol and an overall better clinical outcome, as compared to HPV negative oral squamous cell carcinoma.

This approach is really practical and can be readily implemented in most diagnostic pathology laboratories.

Over expression of p16 has been significantly seen in cases who had history of multiple sexual partners, pan chewing, HIV and tobacco chewing. Findings of high incidence of high risk HPV provide support for a vaccination program for risk group including male population.

Informed education of cases and their close family members and sexual partners will also require knowledge of the HPV status.

HPV prevalence after vaccination with HPV16/18 vaccine was much lower among vaccinated women, suggesting that the vaccine affords strong protection against oral HPV infection, with potentially important implications for prevention of increasingly common HPV- associated oropharyngeal cancer [22].

HPV assessment may play role in early cancer detection, tumour localization, post treatment surveillance and informed consultation of patients and their partners in future.

This type of study must be included in future trials and separate trials in HPV Positive and HPV negative oropharyngeal carcinoma for different treatment outcomes and importance of vaccination programs in men and women of high risk group.

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