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DETECTION OF P^{16INK4A} IN ORAL CAVITY AND OROPHARYNGEAL SQUAMOUS CELL CARCINOMA

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ABSTRACT*: - The incidence of HPV associated Head and Neck Squamous Cell Carcinoma (HNSCC) is increasing over the past 30 years. It is a growing public health concern. It has been reported that tissues of HPV associated HNSCCs over express p16^{INK4a}. Therefore p16^{INK4a} is used as a surrogate marker to detect HPV associated HNSCC. Immunohistochemical detection of p16^{INK4a} is an easy and simple technique than molecular detection of HPVs. Hence we investigated the presence of p16^{INK4a} in Oral and Oropharyngeal Squamous Cell Carcinoma (OPSCC).

***Aims:* The objectives of our study are (1)To study the association of p16^{INK4a} expression with OPSCC, thus with the HPV. (2) To compare the p16^{INK4a}expression in oral cavity and oropharyngeal SCC. (3) To correlate the level of p16^{INK4a} expression with different grades of OPSCC.**

***Methods:* A total sample of 35 cases were analysed during the period of June 2014 to August 2015. We performed IHC in sections of formalin fixed paraffin embedded tissue of OPSCC cases and correlated the various patterns of p16^{INK4a} positivity with respect to histopathological diagnosis.**

***Results:* In the present study, 71.43% of the OPSCC cases were above 50 years of age. OPSCC was more common in males with male to female ratio of 8:1. 94.29% of OPSCC cases were positive for p16^{INK4a}, of which the most common pattern was diffuse nuclear and cytoplasmic staining (37.14%).**

***Conclusion:* In the present study, increased number of OPSCC cases were seen over expressing p16^{INK4a} (94.29%). Oropharynx was the commonest site for p16^{INK4a} positivity (94.44%). Among the oral cavity SCC cases, tongue was the most common site involved (64%). Of the OPSCC cases, most cases (37.14%) had diffuse pattern of p16^{INK4a} over expression. However, DNA detection based studies are needed to validate the utility of IHC detection of p16^{INK4a} as a surrogate marker for HPV associated HNSCC.**

Key words: - Head and neck cancer, HPV, OPSCC, p16^{INK4a}, Squamous cell carcinoma.

INTRODUCTION

Head and Neck Squamous Cell Carcinoma (HNSCC) is the fifth most common cancer worldwide with high incidence of more than 600000 cases every year with high morbidity[1,2]. It causes 200000 deaths annually. The incidence is much higher in India, Southeast Asia and Europe[2].

It is the commonest cancer in males and third most common in females[3]. HNSCC occurs in 5 anatomical sites namely oral cavity, oropharynx, nasopharynx, hypopharynx and larynx[4]. The prevalence of oral SCC in India has shown marked increase in Uttar Pradesh, Madhya Pradesh, Gujarat, Bihar and Maharashtra[5].

There is an association between HPV positive HNSCC with oral sexual behavior, but not in HPV negative HNSCC [6]. Tobacco use is the main cause of HNSCC with as high as 80% of cases attributed to it. Alcohol usage acts synergistically with tobacco in the increased incidence of HNSCC[7]. In the past three decades, there is a decrease in the incidence of HNSCC due to reduction of tobacco use but there is a remarkable increase in the incidence due to HPV infection[8]. Prognosis of $p16^{INK4a}$ positive cases has been reported to be better irrespective of histological grade[9].

The mechanism of HNSCC in HPV negative patients is frequent DNA mutation. HPV positive HNSCC are due to the genetic alterations. The oncogenic proteins E6 and E7 released by the high risk virus subtypes interrupt the p53 and pRb tumor suppressing pathways respectively, which leads to increased cell proliferation and genomic instability leading to carcinogenesis. $p16^{INK4a}$ is one of the several cyclin-dependent kinase inhibitors responsible for regulation of normal cell cycle. As pRb is inactivated by E7 protein, cells are released from growth-suppressive stimuli mediated by the $p16^{INK4a}$. Thus reduced or lost pRb function results in enhanced $p16^{INK4a}$ levels, as a result of a negative feedback control[10]. $p16^{INK4a}$ is commonly used as a biomarker for transcriptionally active HPV-associated cancers[11,12,13].

MATERIALS AND METHODS

Study Place: Department of Pathology, Chengalpattu Medical College and Hospital, Chengalpattu.

Study Design: The present cross-sectional study was a prospective study conducted in the Department of Pathology 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.

Study Population: A total sample of 35 cases of OPSCC was analyzed during the period of June 2014 to August 2015.

Inclusion Criteria: Tissue blocks of patients who are diagnosed as Oral and Oropharyngeal Squamous Cell Carcinoma (OPSCC) by biopsy.

Exclusion Criteria: Tissue blocks of patients who are diagnosed as OPSCC by biopsy and underwent Radiotherapy or Chemotherapy.

Materials used

Tissue sections prepared from paraffin embedded formalin fixed tissues.
Haematoxylin and eosin stain.
$p16^{INK4a}$ monoclonal antibody kit (Mouse monoclonal, Clone (G175-405) ; prediluted).
Positive control included block sections of known $p16^{INK4a}$ positive cases.
Negative control included Primary antibody replaced with PBS and normal oral tissue.

Method:

- Blocks and slides of 35 patients in which histopathological examination of hematoxylin and eosin stained sections of biopsy from Oral cavity and Oropharyngeal sites confirmed as SCC were taken up for the study.
- Immunohistochemistry was performed on the tissue sections taken from the blocks of the cases confirmed as SCC.
- Immunostained sections were reviewed and a strong nuclear as well as cytoplasmic staining was considered as positive reaction, as described by Klaes et al[14].

Distribution of $p16^{INK4a}$ positivity were scored as negative (<1% cells positive), sporadic (<5%

cells positive), focal (<25% cells positive) and diffuse (>25% cells positive) as described by Klaes et al[14].

Data Collection: H &E stained sections and immunostained sections were assessed using light microscope.

Statistical analysis: Datas obtained were coded and entered into the Microsoft excel spread sheet. Datas were compared between groups using Pearson Chi-square or Fisher's exact tests ($p<0.05$). All statistical analysis were performed using SPSS statistical software version 11. Charts were prepared using Microsoft excel 2007.

RESULTS

In the present study 71.43% of the cases of OPSCC were above 50 years of age (Table 1.). However none of the cases were observed below 23 years of age. The youngest age for $p16^{INK4a}$ positive OPSCC cases in our study is 23 years and is 44 years for $p16^{INK4a}$ negative cases. Among the 10 OPSCC cases below 50 years of age, 9 cases (90.00%) were $p16^{INK4a}$ positive. Among the OPSCC cases above 50 years of age 96.00% were positive for $p16^{INK4a}$. The range of the age group is much wider (23-83 years) in the $p16^{INK4a}$ positive cases. The mean age for the $p16^{INK4a}$ positive OPSCC cases is higher (59 years) than $p16^{INK4a}$ negative cases. The median age for $p16^{INK4a}$ negative cases is lower (48 years).

In the sex distribution 88.57% of the OPSCC cases were male and 11.43% of the cases were female (Table 1.). All the 4 female cases (100%) were $p16^{INK4a}$ positive. 29/31 male cases (93.55%) were $p16^{INK4a}$ positive. 3/4 female cases are ≤50 years and all are $p16^{INK4a}$ positive. 7/31 male cases are ≤50 years and 85.71% of which are $p16^{INK4a}$ positive. $p16^{INK4a}$ positive cases are more in the >50 years age groups (72.73%) than in the ≤50 years (Table 1).

Fig.1. Diffuse pattern of $p^{16INK4a}$ immunostaining (x400)

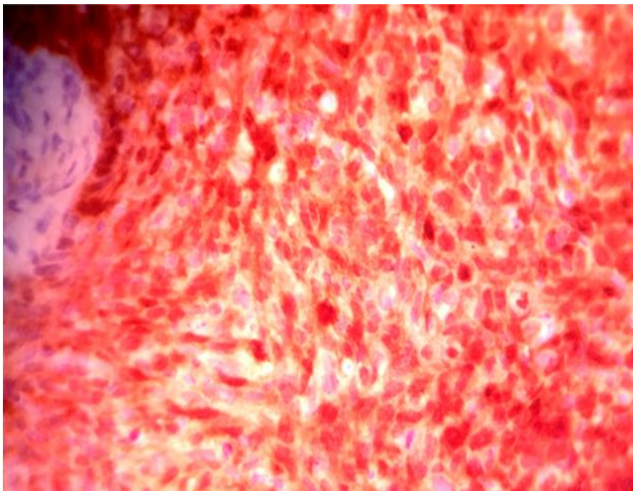

Table 1. Sex distribution of patients with OPSCC in relation to age.

Gender	Total =35	OPSCC									
		Age≤50 years					Age>50 years				
		Total =10	$P16^{INK4a}$ +ve n=9		$P16^{INK4a}$ -ve n=1		Total =25	$P16^{INK4a}$ +ve n=24		$P16^{INK4a}$ -ve n=1	
			n	%	n	%		n	%	n	%
Male	31	7	6	85.71	1	14.29	24	23	95.83	1	4.17
Female	4	3	3	100	0	0	1	1	100	0	0

Oropharynx was most commonly involved by SCC (51.43%) than oral cavity.

Among the oral cavity squamous cell carcinomas, tongue was the most common site involved (64%), followed by buccal mucosa, soft palate, alveolus and gingiva. 90.91% of the tongue squamous cell carcinomas are $p16^{INK4a}$ positive.

Majority of OPSCC cases in our study were of histopathological grade 2 (19/35 cases; 54.29%), followed by grade 1 (14/35 cases; 40%) and grade 3 (2/35; 5.71%) (Table 2, Fig.2.).

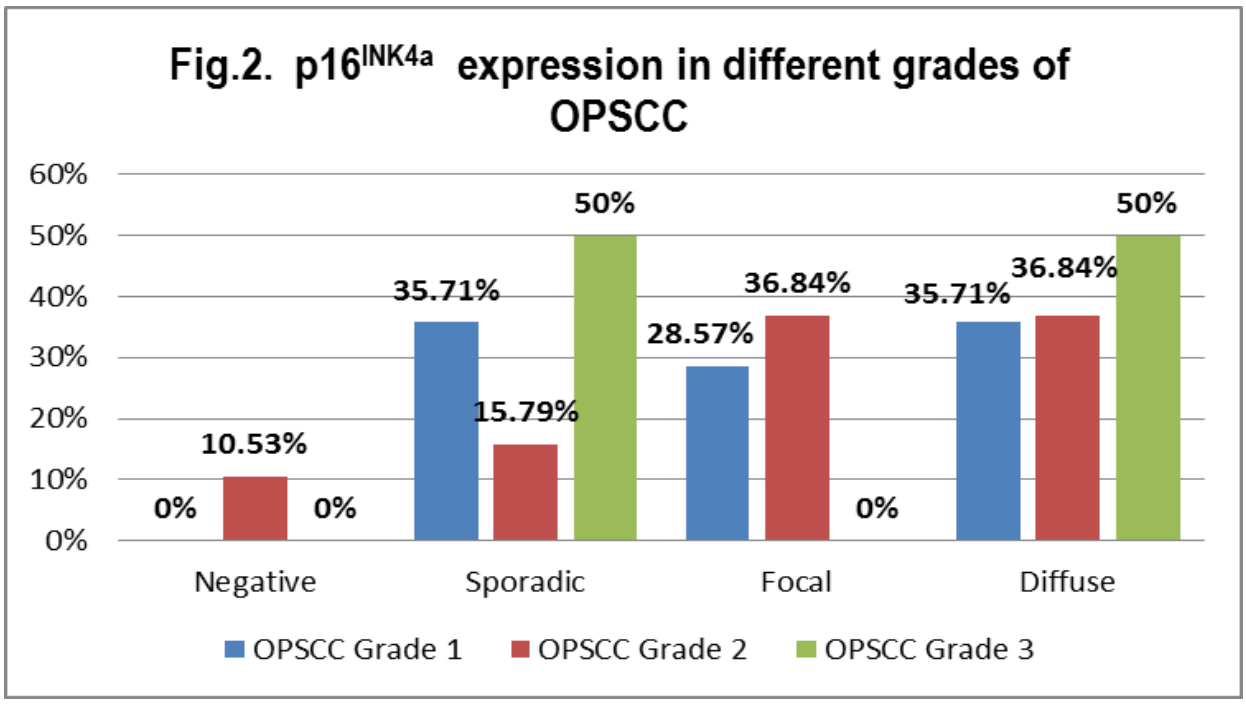

Table 2. Level of $p16^{INK4a}$ expression in different grades of OPSCC

Lesions	Total n=35	Negative n=2		Sporadic n=9		Focal n=11		Diffuse n=13	
		n	%	n	%	n	%	n	%
OPSCC Grade 1	14	0	0	5	35.71	4	28.57	5	35.71
OPSCC Grade 2	19	2	10.53	3	15.79	7	36.84	7	36.84
OPSCC Grade 3	2	0	0	1	50	0	0	1	50
Total	35	2	5.71	9	25.71	11	31.43	13	37.14

While observing the level of expression of $p16^{INK4a}$ by IHC, 94.29% cases of OPSCC were found to be positive (Table 2.). On observing the pattern of expression of $p16^{INK4a}$ in OPSCC, out of 35 cases 37.14% had diffuse pattern, followed by focal (31.43%) and sporadic (25.71%) (Table 2, Fig.2.).

Diffuse pattern of $p16^{INK4a}$ expression were seen in 35.71% (5/14 cases) of grade 1, 36.84% (7/19 cases) of grade 2 and 50% (1/2 cases) of grade 3 OPSCC cases. Sporadic pattern of expression of $p16^{INK4a}$ was observed in 35.71%, 15.79% and 50% of cases among the OPSCC

grade 1, grade 2 and grade 3 respectively (Table 2, Fig.2.).

DISCUSSION

HNSCC continues to be a public health problem with an estimated incidence of 600 000 cases and 200 000 deaths annually[1]. The reports implicating specific HPV types in HNSCC were first published in 1985[15,16]. $p16^{INK4a}$ over expression can be used as a surrogate marker for detection of HPV association in HNSCC.

Our study is a hospital based study and 94.29% cases of OPSCC were positive for the over expression of $p16^{INK4a}$. According to Caihua Liang et al 2012, the prevalence of HNSCC based on PCR and $p16^{INK4a}$ detection based studies was 62%[17].

In the present study 71.43% of the cases of OPSCC are more than 50 years of age. According to Zeyi Deng et al 2014 86.67% cases of HNSCC are more than 50 years of age[18].

In the present study, among the 10 cases (25%) of OPSCC which are less than 50 years of age, 90.00% are $p16^{INK4a}$ positive. According to Zeyi Deng et al 2014 35% of HNSCC are $p16^{INK4a}$ positive[18].

The mean age for $p16^{INK4a}$ positive OPSCC cases in our study is 59 years. According to Zeyi Deng et al 2014 it is 61.8 years[18] for HNSCC and according to Caihua Liang et al 2012 it is56.4 years[17].

The median age for $p16^{INK4a}$ positive and negative OPSCC cases in our study are 60 years and 48 years respectively. According to Gul Kanyilmaz et al 2015 it is 60 and 59 years respectively[19] for HNSCC.

The range of the age group for $p16^{INK4a}$ positive OPSCC cases in our study is 23 to 83 years. In comparison, study by Gul Kanyilmaz et al 2015 and Zeyi Deng et al 2014 had 15 to 70 years and 39 to 89 years respectively for HNSCC[18,19].

The youngest age was 23 years among the $p16^{INK4a}$ positive OPSCC cases, while it was 39 years for HNSCC by Zeyi Deng et al 2014[18].

In our study, male cases among the total OPSCC cases is 88.57% correlating with Gul Kanyilmaz et al 2015 which is 88.55% for HNSCC[19].

In our study of OPSCC, 93.55% male patients and 100% female patients are $p16^{INK4a}$ positive. According to Gul Kanyilmaz et al 2015 40.52% male patients and 73.33% female patients are $p16^{INK4a}$ positive[19] for HNSCC. According to Zeyi Deng et al 2014 18.9% male patients and 26.1% female patients are $p16^{INK4a}$ positive[18]. According to Caihua Liang et al 2012 27.7% male patients and 11.9% female patients are $p16^{INK4a}$ positive[17].

In our study, more than 50 years age group constituted the major population with 25 cases, out of which 24 cases (96%) showed $p16^{INK4a}$ positivity. 10 cases were less than 50 years age group with (9/10 cases) 90% showing $p16^{INK4a}$ positivity (Table 1.).

In our study, oropharynx is the most common site followed by oral cavity which is correlating with Zeyi Deng et al 2014[18].

The percentage of $p16^{INK4a}$ positive cases is highest (94.44%) in the oropharynx and lowest (94.12%) in the oral cavity correlating with Zeyi Deng et al 2014, where oropharynx was 37.7% and oral cavity 8.3%[18].

In our study, in the oral cavity squamous cell carcinoma, the most common site involved is tongue (64%) and the least common site involved is the alveolus and gingiva (each 6%). According to Pradyot Prakash et al 2013 the most common

site is the tongue (37.7%) and the least common site is the hard palate and gingiva (each 1.45%)[20].

In our study, 90.91% of tongue squamous cell carcinomas were $p16^{INK4a}$ positive.

In our study, $p16^{INK4a}$ positive cases are highest (100%) in the grade 3 and grade 1 OPSCC, followed by grade 2(89.47%) contradicting with Zeyi Deng et al 2014, where highest (42.1%) in the grade 1 HNSCC, followed by grade 2(19%) and lowest (14.7%) in the grade 3 HNSCC[18].

In our study, in the oral cavity squamous cell carcinoma, most (35.30%) are having diffuse pattern, followed by focal and sporadic patterns, each having 29.41%. In comparison with Pradyot Prakash et al 2013, in the oral cavity squamous cell carcinoma, diffuse pattern is the most common (31.9%), followed by sporadic (24.6%) and lowest (14.5%) having focal pattern of expression[20]. Similarly diffuse positivity was the commonest pattern observed even among the OPSCC (37.14%).

CONCLUSION

The present study demonstrated increased association of $p16^{INK4a}$ over expression in cases of OPSCC (94.29%). OPSCC was more common in males with male to female ratio of 8:1. Oropharynx accounted for the most common site (51.43%) than oral cavity. Also oropharynx was the most common site for $p16^{INK4a}$ positivity in OPSCC cases (94.44%).

Among the oral cavity SCC cases, tongue was the most common site involved (64%). Among the $p16^{INK4a}$ positive cases most are OPSCC Grade 2 (51.52%). Of the OPSCC cases, most cases (37.14%) had diffuse pattern of $p16^{INK4a}$ over expression. Diffuse pattern of $p16^{INK4a}$ over expression was most common in OPSCC Grade 2 cases (53.85%).

Further, DNA detection based studies are needed to validate the utility of IHC detection of $p16^{INK4a}$ as a surrogate marker for HPV associated OPSCC.

In future, prophylactic vaccination for boys and girls before the starting of sexual activity will prevent HPV infection and thus reduce the incidence of HPV associated HNSCC. Plans to improve public awareness and knowledge of clinical features and risk factors will reduce the disease burden of HPV associated HNSCC.

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