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Detection of $P^{16ink4a}$ in Non-Oro pharyngeal Head and Neck 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 Non-Oropharyngeal (nasopharynx, hypopharynx and larynx)Squamous Cell Carcinoma (Non-OPSCC).*

Aims :*The objectives of our study are (1)To study the association of $p16^{INK4a}$ expression with Non-OPSCC, thus with the HPV. (2)To compare the $p16^{INK4a}$ expression in different subsites of the Non-OPSCC. (3)To correlate the level of $p16^{INK4a}$ expression with different grades of Non-OPSCC.*

Materials and methods :*A total sample of 25 cases were analysed during the period of June 2014 toAugust 2015. We performed IHC in sections of formalin fixed paraffin embedded tissue of Non-OPSCC cases and correlated the various patterns of $p16^{INK4a}$ positivity with respect to histopathological diagnosis.*

Results :*In the present study, 80.00% of the Non-OPSCC cases were above 50 years of age. Non-OPSCC was more common in males with male to female ratio of 4:1 . 92.00% of Non-OPSCC cases were positive for $p16^{INK4a}$, of which the most common pattern was diffuse nuclear and cytoplasmic staining (76.00%).*

Conclusion :*In the present study, increased number of Non-OPSCC cases was seen over expressing $p16^{INK4a}$ (92.00%). Hypopharynx was the commonest site for $p16^{INK4a}$ positivity (92.30%). Of the Non-OPSCC cases, most cases (76.00%) 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, Non-OPSCC, $p16^{INK4a}$, Squamous cell carcinoma*

I. 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]. There is an association between HPV positive HNSCC and oral sexual behavior, but not in HPV negative HNSCC[5] . 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[6]. In the past three decades, there is a decrease in the incidence of HPV negative HNSCC due to reduction of tobacco use but there is a remarkable increase in the incidence of HNSCC due to HPV infection[7] .Prognosis of $p16^{INK4a}$ positive cases has been reported to be better irrespective of histological grade[8].

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[9]. $p16^{INK4a}$ is commonly used as a biomarker for transcriptionally active HPV-associated cancers[10,11,12].

II. 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 25 cases of Non-OPSCC was analyzed during the period of June 2014 to August 2015.

Inclusion Criteria: Tissue blocks of patients who are diagnosed as Non-Oropharyngeal (nasopharynx, hypopharynx and larynx) Squamous Cell Carcinoma (Non-OPSCC) by biopsy.

Exclusion Criteria: Tissue blocks of patients who are diagnosed as Non-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 25 patients in which histopathological examination of hematoxylin and eosin stained sections of biopsy from Non-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 Klaeset al[13].

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[13] .

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 was performed using SPSS statistical software version 11. Charts were prepared using Microsoft excel 2007.

III. Results

In the present study 80% of the cases of Non-OPSCC were above 50 years of age (Table 1.). However none of the cases were observed below 22 years of age .The youngest age for $p16^{INK4a}$ positive Non-OPSCC cases in our study is 22 years and is 60 years for $p16^{INK4a}$ negative cases. Among the 5 Non-OPSCC cases below 50 years of age, all cases (100%) were $p16^{INK4a}$ positive. Among the Non-OPSCC cases above 50 years of age 90.00% were positive for $p16^{INK4a}$. The range of the age group is much wider (22-80 years) in the $p16^{INK4a}$ positive cases. The mean age for the $p16^{INK4a}$ positive Non-OPSCC cases is lower (58 years) than $p16^{INK4a}$ negative cases. The median age for $p16^{INK4a}$ positive cases is lower (61 years).

In the sex distribution 80.00% of the Non-OPSCC cases were male and 20.00% of the cases were female (Table 1.). All the 5 female cases (100%) were $p16^{INK4a}$ positive. 18/20 male cases (90.00%) were $p16^{INK4a}$ positive. 2/5 female cases are ≤50 years and all are $p16^{INK4a}$ positive. 3/20 male cases are ≤50 years and all (100%) are $p16^{INK4a}$ positive. $p16^{INK4a}$ positive cases are more in the >50 years age groups (78.26%) than in the ≤50 years (Table **1)Hypopharynx**was most commonly involved by SCC (52.00%) than Larynx. Majority of Non-OPSCC cases in our study were of histopathologicalgrade 2 (16/25 cases; 64.00%), followed by grade 1 (9/25 cases; 36.00%) (Table 2, Fig.2.).While observing the level of expression of $p16^{INK4a}$ by IHC, 92.00% cases of Non-OPSCC were found to be positive (Table 2.). On observing the pattern of expression of $p16^{INK4a}$ in Non-OPSCC, 19/25 cases (76.00%) had

diffuse pattern, followed by focal (12.00%) and sporadic (4.00%) (Table 2, Fig.2.).Diffuse pattern of $p16^{INK4a}$ expression were seen in 88.89%**(8/9 cases)of**grade 1 and 68.75% (11/16 cases) of grade 2 Non-OPSCC cases.3/25 cases had focal pattern of expression of $p16^{INK4a}$ and all were of grade 2. (Table 2, Fig.2.).

IV. 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[14,15]. $p16^{INK4a}$ over expression can be used as a surrogate marker for detection of HPV association in HNSCC. Our study is a hospital based study and 92.00% cases of Non-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%[16]. In the present study 80% of the cases of Non-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[17].In the**present study, among the 5 cases (25%) of Non-OPSCC which are less than 50 years of age, 100.00% are $p16^{INK4a}$ positive. According to Zeyi Deng et al 2014 35% of HNSCC are $p16^{INK4a}$ positive[17].The mean age for $p16^{INK4a}$ positive Non-OPSCC cases in our study is 58 years. According to Zeyi Deng et al 2014 it is 61.8 years[129] for HNSCC and according to Caihua Liang et al 2012 it is56.4 years[16].The median age for $p16^{INK4a}$ positive and negative Non-OPSCC cases in our study is 61 years and 70 years respectively. According to GulKanyilmaz et al 2015 it is 60 and 59 years respectively[18]for **HNSCC.The**range of the age group for $p16^{INK4a}$ positive Non-OPSCC cases in our study is 22 to 80 years. In comparison, study by GulKanyilmaz et al 2015 and Zeyi Deng et al 2014 had 15 to 70 years and 39 to 89 years respectively for HNSCC[18,17]. The youngest age was 22 years among the $p16^{INK4a}$ positive Non-OPSCC cases, while it was 39 years for HNSCC by Zeyi Deng et al 2014[17].In our study, male cases among the total Non-OPSCC cases is 80.00% correlating with Zeyi Deng et al 2014 which is 84.67% for HNSCC[17].

In our study of Non-OPSCC, 90.00% male patients and 100% female patients are $p16^{INK4a}$ positive. According to GulKanyilmaz et al 2015 40.52% male patients and 73.33% female patients are $p16^{INK4a}$ positive[18]for HNSCC. According to Zeyi Deng et al 2014 18.9% male patients and 26.1% female patients are $p16^{INK4a}$ positive[17]. According to Caihua Liang et al 2012 27.7% male patients and 11.9% female patients are $p16^{INK4a}$ positive[16].

In our study, more than 50 years age group constituted the major population with 20 cases, out of which 18 cases (90%) showed $p16^{INK4a}$ positivity. 5cases were less than 50 years age group, with all cases (100%) showing $p16^{INK4a}$ positivity **(Table 1).In our**study, hypopharynx is the most common site followed by larynx which is correlating with Zeyi Deng et al 2014[17]. We did not receive specimens from nasopharynx. Nasopharynx is the least common site **involved.The**percentage of $p16^{INK4a}$ positive cases is highest (92.30%) in the hypopharynx and lowest (91.67%) in the larynx **contradictingwith**Zeyi Deng et al 2014, where hypopharynx was 7.7% and larynx 12.5%[17].

In our study, $p16^{INK4a}$ positive cases are highest (64.00%) in the grade 2 Non-OPSCC, followed by grade 1 (36.00%) contradicting with Zeyi Deng et al 2014, highest (42.1%) in the grade 1 HNSCC, followed by grade 2(19%) and lowest (14.7%) in the grade 3 HNSCC[17].In our study, among the Non-OPSCC cases, most (76.00%) are having diffuse pattern of $p16^{INK4a}$ expression, followed by focal (12.00%) and lowest (4.00%) having sporadic pattern of expression.

V. Conclusion

The present study demonstrated increased association of $p16^{INK4a}$ over expression in cases of Non-OPSCC (92.00%). Non-OPSCC was more common in males with male to female ratio of 4 : 1 . Hypopharynx accounted for the most common **site (52.00%)than**larynx. Also hypopharynx was the **mostcommon**site for $p16^{INK4a}$ positivity in Non-OPSCC cases (92.30%).Among the $p16^{INK4a}$ positive cases most are Non-OPSCC Grade 2 (65.22%). Of the Non-OPSCC cases, most cases (76.00%) had diffuse pattern of $p16^{INK4a}$ over expression. Diffuse pattern of $p16^{INK4a}$ over expression was most common in Non-OPSCC Grade 2 cases (57.89%).

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

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

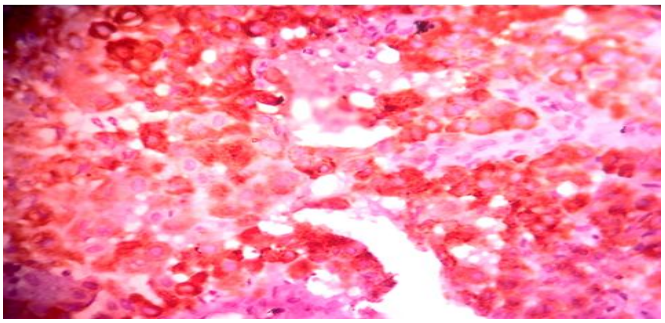

Fig.2. p16^{INK4a} expression in different grades of Non-OPSCC

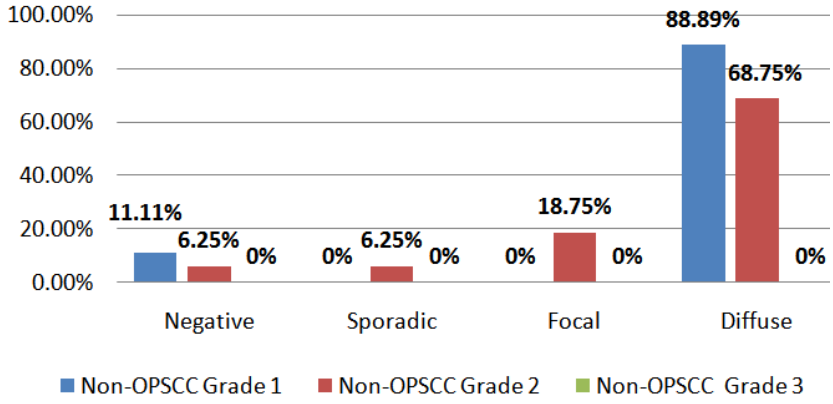

Table 1.Sex distribution of patients with Non-OPSCC in relation to age.

Gender	Total =25	Non-OPSCC									
		Age≤50 years					Age>50 years				
		Total =5	P16^{INK4a} +ve n=5		P16^{INK4a} -ve n=0		Total =20	P16^{INK4a} +ve n=18		P16^{INK4a} -ve n=2	
			n	%	n	%		n	%	n	%
Male	20	3	3	100	0	0	17	15	88.24	2	11.76
Female	5	2	2	100	0	0	3	3	100	0	0

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

Lesions	Total n=25	Negative n=2		Sporadic n=1		Focal n=3		Diffuse n=19	
		n	%	n	%	n	%	n	%
Non-OPSCC Grade 1	**9**	**1**	**11.11**	**0**	**0**	**0**	**0**	**8**	**88.89**
Non-OPSCC Grade 2	**16**	**1**	**6.25**	**1**	**6.25**	**3**	**18.75**	**11**	**68.75**
Non-OPSCC Grade 3	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Total	25	2	8.00	1	4.00	3	12.00	19	76.00

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