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ORIGINAL RESEARCH

Evaluation of p16 and p53 expression in squamous cell carcinomas of pyriform fossa in comparison with those in oropharynx and oral cavity in an Indian population

[1]Dr. Thongam Sachin Singh, [2]Dr. Venkatesan Somasundaram, [3]Dr. Barun Kumar Chakrabarty, [4]Dr. Bhupendra Prakash Solanke, [5]Dr. Nilanjan Roy

[1]Assistant Professor, [2]Professor & HOD, [3]Associate Professor, Department of Pathology, Armed Forces Medical College, Pune India
[4]Assistant Professor, Department of Pharmacology, Armed Forces Medical College, Pune India
[5]Professor, Department of Surgery, Armed Forces Medical College, Pune India

Corresponding author
Dr. Thongam Sachin Singh
Assistant Professor, Department of Pathology, Armed Forces Medical College, Pune India
Email: sachinthongam@gmail.com

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ABSTRACT

Background: Prevalence of HPV in oropharyngeal squamous cell carcinomas is well documented however few studies had evaluated it in pyriform fossa. This study was aimed to evaluate expression of p16 in squamous cell carcinomas of pyriform fossa as compared to oropharynx and oral cavity. **Methods:** 148 cases of squamous cell carcinomas of head and neck diagnosedonly on biopsies were evaluated for p16 and p53 expression in tertiary care centre.Correlation with demographic parameters andhistological grade performed. **Results:** In overall, p16 was positive in 67 cases (45.27%) and p53 in 109 cases (73.7%). p16 was positive in 17 /36 cases in pyriform fossa (47.22%), 47/62 cases in in oropharynx (70.1%) and 3/50 in oral cavity (4.47 %). p53 was positive in 30/36 cases in pyriform fossa (83.33%), 34/62 cases in oropharynx (54.83%) and 45/50 cases in oral cavity (90%). Mean age was 60.61yearsand medianof 62 years. M:F ratio was 6:1. Well differentiated SCCs showed maximal p53 positivity whilemoderately and poorly differentiated SCCs showedmixed p16 and p53. 11/25 (44%) MDSCC K and 5 out 6 MDSCC-NK cases in pyriform fossa expressed p16 while 20/ 25(80%) MDSCC Kand 6/6 MDSCC NKcases in pyriform fossa were p53 positive,Significant association of p16was found with tumour site, grade, and smoking (p value <0.05) but not with age, gender,and alcohol. **Conclusions:** Squamous cell carcinoma of pyriform fossa expressedp16 significantlyas compared to those in oropharynx. p53 expression is a constant finding at all sites.

Keywords: Squamous cellcarcinoma, Human papilloma virus, Immunohistochemistry, p16, p53, Pyriform fossa, Oropharynx

INTRODUCTION

According to GLOBOCON 2022, of all cancers, hypopharyngeal cancers stood 25th in both incidence and mortality, whereas oropharynx cancers stood 24th in incidence and 23th in mortality respectively. Lip and oral cavity stood 16th in incidence and 15th in mortality. Squamous cell carcinomas are the commonest type of cancers in these locations.[1] Traditionallymajority of head and neck SCCs are thought to be related to tobacco use, alcohol abuse or both but role of prior infection with oncogenic strain of HPV are increasing. Because of its role in the carcinogenesis HPV infection especially sub-types 16 and 18, enjoyed greater importance in oropharyngeal SCCs however it is worth noting that low risk HPV infections are very common in larynx especially in papillomatosis.Study by Juan P et al in Spain on 124 patients surgically treated for laryngeal (62 cases) and hypopharyngeal (62 cases) SCC using p16 IHC and PCR based DNA detection found 14% patients positive for p16 and concluded that occasionally HPV is involved in laryngeal and hypopharyngeal SCCs.[2]Gholap Devyani et al studied the prevalence of HPV types in Indian population in different subsites of Head and neck cancers and found 38.89 % positive p 16 in hypopharyngeal cancers even though the total number of these cancers was less (only 18 cases).[3]Lingen MW, Xiao W et al. and LeeSY Choi

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EC et al. also reported that in non-oropharyngeal sites rate of HPV infections are substantially lower (1.3-7%).[4,5]Combes JD and Franceschi S on their review article on role of HPV on non-oropharyngeal head and neck cancers suggested that HPV does play as significant risk factor for hypopharyngeal cancers.[6]

It is well known that pathogenesis of HPV infection involves inactivation of p53 and retinoblastoma protein (RB) tumour suppressor genes by HPV E6 and E7 oncoproteins respectively. Expression of E7 viral oncoprotein inactivates retinoblastoma proteins (pRb), which consequently increases the E2F activation factors in the cell leading to increased p16 in the S-phase of the cell cycle.[7,8] Fakhry C and Psyrri A et al. reported that HPV oropharyngeal cancers is associated with younger age, male gender, multiple sexual partners, and higher socio-economic status.[9]Whether same demographic profiles apply to non-HPV cancers is unclear.Windon MJ, D'Souza G et al. reported that non-HPV cancers are likely in smokers, persons with multiple co- morbidity and less sexual partners.[10]

As it could be seen from the above facts that few studies addressed the relation of HPV in non-oropharyngeal SCCs and there are wide variation of its prevalence ranging from 1.3- 38.89% of p16 positive SCCs in these locations globally. In view of this varied consensus and to fill in the gap in knowledge regarding HPV infection in hypopharyngeal region especially pyriform sinus this study was planned to understand its prevalence and significance of HPV infection in non-oropharyngeal location especially pyriform fossa by doing comparative study of p16 and p53 expression in SCCs of various subsites of head and neck such as oral cavity, oropharynx diagnosedon biopsies only. Few demographic parameters and histological grade of these carcinomas were studied to see their association.

MATERIAL AND METHODS

A retrospective and prospective cross-sectional study was conducted on 148 cases of squamous cell carcinomas diagnosed on biopsies over the last 4 years in a tertiary care centre of northern India.

Inclusion criteria: All cases of SCCs diagnosed on biopsies from hypopharynx, especially pyriform fossa, oropharynx, and oral cavity over the period of 4 years.

Exclusion criteria: Inadequate or absent residual tissue on FFPE blocks

Fresh biopsy specimens with clinical diagnosis of squamous cell carcinoma of oral cavity, oropharynx, and pyriform fossa were fixed in 10% formalin and processed after 24 hours. Sites of biopsy in the current study included oral cavity comprised tongue, floor of mouth, alveolar margin, buccal mucosa; base of tongue and tonsillar area in oropharynx and pyriform fossa in hypopharynx.Haematoxylin and Eosin staining were performed on all tissue sections. Old formalin fixed paraffin embedded blocks of all diagnosed cases of squamous cell carcinoma of head and neck were also retrieved and their diagnosis confirmed. Relevant clinical data were also collected from pathological reports and clinical cases sheets.

Immunochemistry tests for p16 and p53 were performed on 1-2 sections of 3-4 microns thickness mounted on poly-l-lysine slides. Mouseantihuman p16 oncoprotein (INK4) (g175-405) manufactured by Vitro master Diagnostica, Sevilla Spain with positive control from tonsillar tissue and mouse antihuman p53 protein, do-7(Dako-n1581) with positive control of tissue from colon carcinoma were used. p16 or p53 was considered positive when more than 50 % of the tumour cells showed both nuclear and cytoplasmic staining.

Data analysis: Clinical and histopathological data wereentered onMicrosoft excel sheet and analysed using SPSS. p value of less than 0.05 was considered significant.

Ethical Statement: No patient was treated with any non-standard or experimental therapy. Approval from institutional ethical and scientific committee has been obtained.

RESULTS

148 punch biopsies cases of SCC from oral cavity, oropharynx and hypopharynx were studied.

Table 1. Distribution of cases based on sites

Site	Number of cases	p16(+)	p16(-)	p 53(+)	p53 (-)	p value
Hypopharynx (n=36)		17	19	30	6	
Pyriform Fossa	36					
Oropharynx(n=62)						
Base of Tongue	50	35	15	27	23	p16 <.001
Tonsillar area	12	12	0	6	6	p53 = .003
Oral cavity (n=50)						
Alveolus	9	2	7	8	1	
Buccal mucosa	13	1	12	12	1	
Floor of mouth	4	0	4	4	0	
Hard palate	3	0	3	3	0	
Retromolar trigone	4	0	4	4	0	

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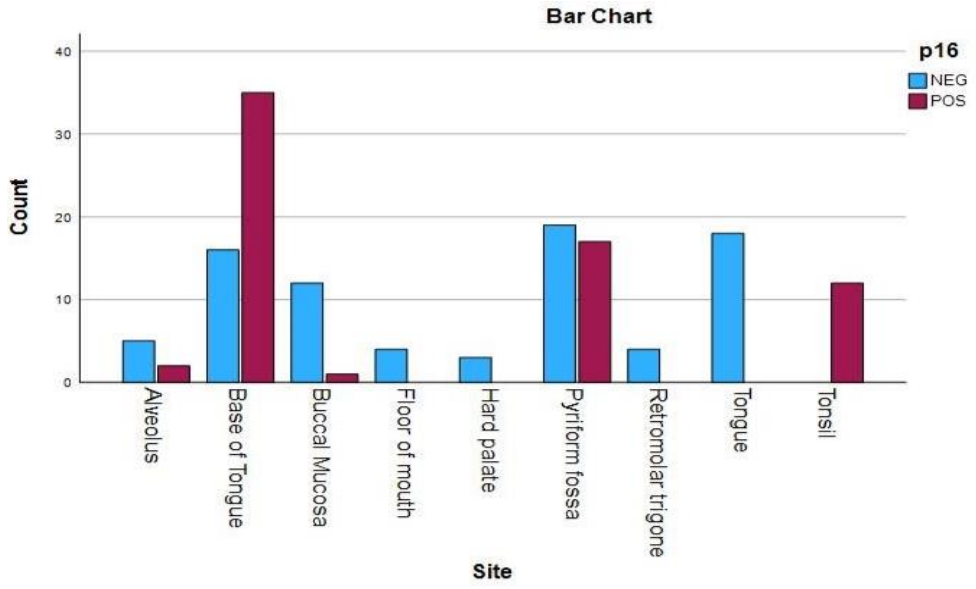
Tongue	**17**	0	17	15	2	
	Total=148	67	81	109	39	

p16 and p53 expression studied were found significantly associated with subsites studied. 47.22% of all cases in pyriform fossa were positive for p16 when 75.8 % were positive in oropharynx. Similarly, 83.33 % of all cases in pyriform fossa were positive for p53 when 53.22 % were positive in oropharynx. 92% of cases in oral cavity were positive for p53.Bar charts 1 and 2 showed p16 and p53 expression based on different sub-sites.



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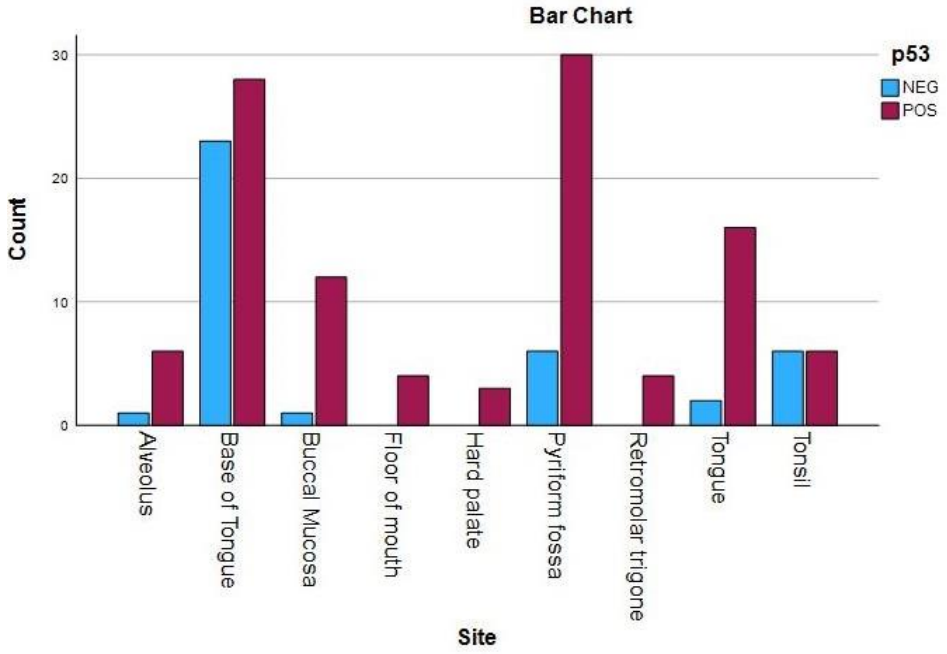

(b)

Figure 1. Bar charts p16 (a) and p53 (b) expression in different sub-sites.

Table 2 and 3 given below showeddemographic parameters and grade with p16 and p53 expressions.

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Table 2. Comparison of p16 expressed on different sites based on demographic parameter and grade of the carcinomas

Parameter		No of cases	Pyriform fossa (n=36)		Oropharynx (n= 62)		Oral cavity (n=50)		P value
			P 16 Positive	P 16 Negative	P 16 Positive	P 16 Negative	P 16 Positive	P 16 Negative	
Age(yrs)	20-40	7	1	0	1	1	0	4	p16 =0.188
	41-60	62	8	8	26	2	2	16	
	61-80	79	8	11	20	12	1	27	
Gender	Male	127	16	18	42	10	2	39	p16 =0.235
	Female	21	1	1	5	5	1	8	
Smoking	YES	104	10	19	22	12	2	39	p16 = <.001
	OCC	27	4	0	21	0	0	2	
	NO	17	3	0	4	3	1	6	
Alcohol	Yes	92	12	17	19	10	1	33	p16 =0.361
	Occasional	21	1	1	17	0	0	2	
	No	35	4	1	11	5	2	12	
Grade	WDSCC	29	1	4	1	2	1	20	p16 <0.001
	MDSCC K	85	11	14	25	12	0	23	
	MDSCC NK	17	5	1	8	1	1	1	
	PDSCC	17	0	0	13	0	1	3	

Table 3. Comparison of p53 expressed on different sites based on demographic parameter and grade of the carcinomas

Parameter		No of cases	Pyriform fossa (n=36)		Oropharynx (n= 62)		Oral cavity (n=50)		P value
			P 53 Positive	P 53 Negative	P 53 Positive	P 53 Negative	P 53 Positive	P 53 Negative	
Age(yrs)	20-40	7	0	1	1	1	4	0	p53= 0.885
	41-60	62	13	3	15	13	16	2	
	61-80	79	17	2	17	15	26	2	
Gender	Male	127	28	6	26	26	38	3	
	Female	21	2	0	7	3	8	1	p53=0.461
Smoking	YES	104	24	5	19	15	40	1	
	OCC	27	3	1	10	11	2	0	p53=0.026
	NO	17	3	0	4	3	4	3	
Alcohol	Yes	92	24	5	16	13	33	1	
	Occasional	21	2	0	9	8	2	0	p53=0.548
	No	35	4	1	8	8	11	3	
Grade	WDSCC	29	4	1	3	0	19	2	p53 =0.091
	MDSCC K	85	20	5	20	17	22	1	
	MDSCC NK	17	6	0	3	6	1	1	
	PDSCC	17	0	0	7	6	4	0	

Mean age of patients in the study is 60.61+_ 10.58 years. Same median and mode value of 62 years was observed. No significant association found with expression of both markers.M:F ratio in the study was 6:1 but no significant association was found with both markers.Most males smoked while, 11 women had a history of regular smoking. 10/104 patients who smoked regularly showed p16 positive in pyriform fossa while 22/104 in oropharynx showed p16 positivity. Therewas asignificant association between smokingand expression of p16 or p53.Similarly, most males consumed while no women consumed alcohol in the present study. But no association with either marker observed.

In the present study those patients with history of occasional smoking or alcohol consumption showed maximum p16 positivity in oropharyngeal SCCs. Same was not true for p53 expression.Only 18/148 cases were documented with history of either obesity, diabetes, hypertension, or CAD but no significant association.

p16 positives in WDSCCS were negligible but maximum negatives in oral cavity. As expected, p53 positives WDSCCs were maximum in oral cavity (19/29 cases).

Moderately differentiating SCCs with or without keratinisation were maximally found in oropharynx followed by pyriform fossa.They showed significant variable expression of both p16 and p53 in these sites, however those MDSCCs in pyriform fossa showed maximum positivity of p53. Most of poorly differentiated SCCs (17 cases) were in oropharynx

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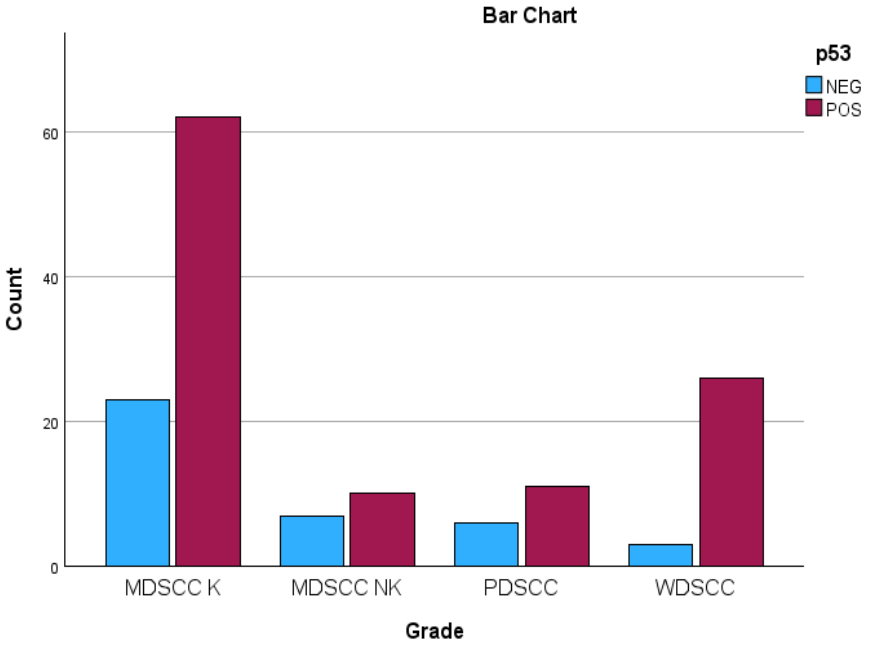
and majority showed positive p16. No cases of PDSCCs were found in pyriform fossa

Significant association of grades with p16 expression ($p < 0.001$) was observed but not with p53($p = 0.091$) expression. Moderately differentiated SCCsshowed maximum p16 and p53 expression in oropharynx and pyriform fossa. Poorly differentiated carcinoma showed p16 expression especially in oropharynx. Bar charts showing grade and p16 and p53 expression.and photomicrographs of H& E sections of SCC and itsp16 immunostaining are also produced.



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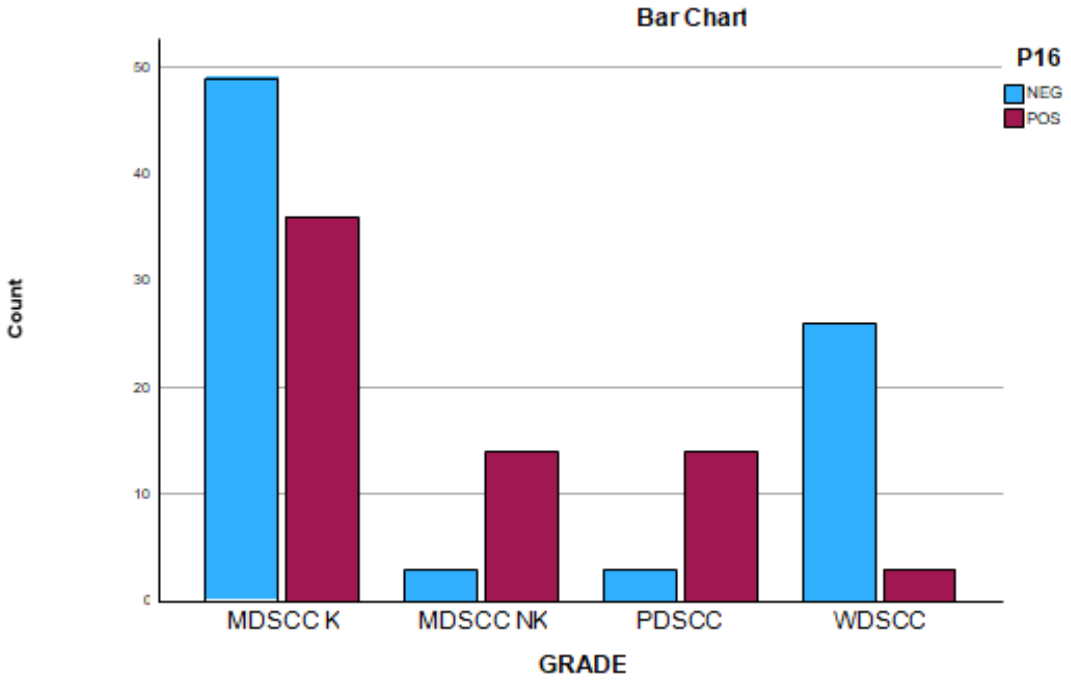

(b)

Figure 2. Bar charts showing p16(a) and p53(b) status as per grades

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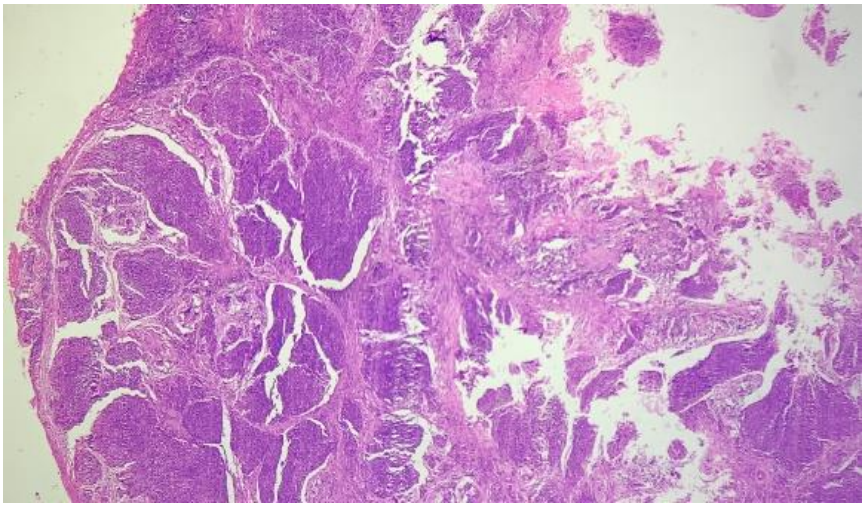

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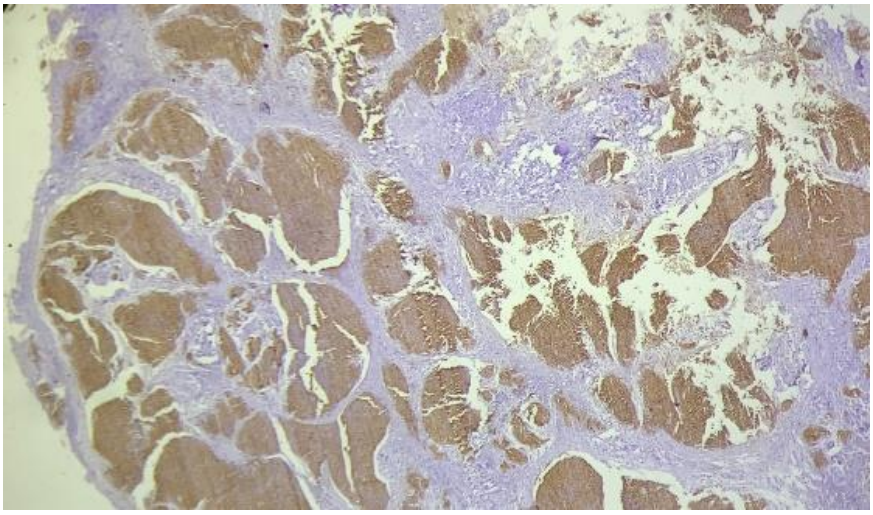

B

Figure 3. Photomicrographs of nonkeratinizing SCC. A. H& E 20X B. IHC p16, 20X

DISCUSSION

According to literature, the prevalence of p16 positive SCC in hypopharynx especially pyriform fossa is relatively low. Salazar CR et al. [11]reported 8% p16 positive hypopharyngeal SCC in study involving 163 tumour specimens with maximum positivity in oropharynx (53%). There are also studies like that of Pernille Lassen et al.[12] which reported 14 % positive in hypopharynx SCC compared to 52% in oropharynx.

In the Indian context, study by Gholap Devyani et al.[3] on the studies to know the prevalence of HPV infection types in cancers in different subsites of Head and Neck reported prevalence of 30.89 % p16 positive SCCs in hypopharynx though the number of cases was less (18 cases only.

In the present study, prevalence of HPV in pyriform fossa SCCs is 47.2% (17 out 36 cases) which is much greater than the previous study done on Indian population. On the other hand, prevalence of p53 positive SCCs in pyriform fossa is 83.33 % (30/36 cases). Peak incidence of hypopharyngeal SCCs occurred in people aged between 50 and 60 years.[13]

Lassen et al.[14]and Murthy et al.[15]in their studies on head and neck cancers noted that p16 positivity was seen in patient of 57 and 53 years, respectively when compared to p16 negative ones (60 and 57 years, respectively. In the current study average age for non-HPV cancers was 63 years while for HPV cancers it was 62 years. No significant difference of age was found between Oropharyngeal and Pyriform fossa SCCs in the present study as maximum cases were above 60 years at all sites.

In the current study males outnumber the females by a ratio of 6:1. p16 positive cases amongst males in pyriform fossa (44.4%)and oropharynx (67.7%) were observed. Carol Chelimo and J mark Elwood on their study to determine sociodemographic differences amongst head and neck cancers reported male: female ratio of 5.75 for hypopharyngeal SCCs.

There are other studies conducted by Meng et al.[17] and Sritippho et al.[18] who also documented HPV or p16 expression to be associated with male sex. Similarly studies by Murthy et al. [15]and Ralli M et al. [19]also male sex to be favoured for HPV infection but no significant statistically.Males may be at increased

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risks for both non-HPV and HPV cancers due to more consumption of tobacco or alcohol or increased exposure to oral sex. On the contrary Baiker et al. [20[and Shinoharaet al. [21] noted it to be more common amongst females. However, no association of HPV infection or p16 expression with regards to genderor age was found in all locations.This agreed with study conducted by Singh et al. [22] who did not find any association of p 16 with age or sex too.

Heavy users of tobacco and alcohol have a more than 35-fold higher risk of developing HNSCC. [23]Upper hypopharyngeal SCCs i.e pyriform fossa are more associated with heavy alcohol consumption and smoking.[13]In the current study, greater number of the patient who consumed alcohol did show higher p16 and p53 positivity but did not any significant association. However, those who smoked regularly showed higher p16 expression in SCC over and above p53 positivity. Those patients who both smoked and consumed showed higher rate of p16 positivity in pyriform and oropharynx but not in oral cavity while p53 positivity is increased all sites in this study. It is believed that due to cultural reasons and addictive nature, both habits are closely linked and their effects synergistic.[24]This can be explained by the fact the synergistic action of alcohol in promoting carcinogenesis. In fact, alcohol can serve as a solvent for tobacco carcinogens such as polyaromatic hydrocarbons and nitrosamines which enable enhanced exposures epithelial cells to these substances.[25]Consuming both substances together significantly increase the risk of developing cancer compared to using either one alone, essentially multiplying the risk due to a combined effect where each substance enhances the harmful effects of the other; this is often described as a "multiplicative" interaction where the combined risk is greater than the sum of individual risks. [26,27]

In the present study, 67 out 148 SCC cases (45.25%) showed p16 which signified a fairly high rate of incidence of HPV associated carcinoma in upper aero-digestive locations. p16 was significantly associated with site(p value <0.001) and so did p53 (p value = 0.003) p16 was maximally positive in oropharynx (47cases) followed by pyriform fossa cancers (17cases). P53 expression was seen in 109 cases with maximum positivity in oral cavity (45) followed by oropharynx (34) and pyriform fossa (30). These findings agreed with those studies by Singh et al. [22]and Lechner M et al. [28] al who documented that HPV positive cases were mainly in oropharynx especially base of tongue and tonsil. p16 positivity in oral cavity SCC was none in a study by Mishra A et al. [29]which almost collaborated with the present study where it was also almost 100 % negative in oral cavity cancers (47cases) except 3 cases). It is interesting to note that just more 50% of cases from pyriform fossa (52.7%) showed negative p16 while majority expressed p53(83.3%).

In the present study it was found that well differentiated SCC are predominantly in oral cavity and showing maximum p53 positivity (26 out 29 cases); only three were positive for p16 in oral cavity cancers.In study conducted by Babiker et al. [20]they found significant association of p53 in OSCC cases (54.5%) which agrees with present study (65.5%). One of 4 WDSCC in pyriform fossa showed p16 positive.

Chernock RD et al. [30] documented that non keratinising is the most common histology in SCCS related to HPV, more in oropharynx. In the current study, 14 out 17 MDSCC-NK (82.35%) and 36 out 85 MDSCC K (42.35%) showed p16 positivity. On the other hand, 10 out of 17 and p53(59%) and 62 out of 85 showed p53 expression (73%) showed p53. 11/25 (44%) MDSCC K and 5 / 6 MDSCC-NK cases (83.33%) in pyriform fossa expressed p16 while 20/ 25(80%) MDSCC K and 6/6 MDSCC NK cases (100%) in pyriform fossa were p53 positive. Moderately differentiating SCCs were greater in number in oropharynx followed by pyriform fossa and oral cavity. Association of p16 in MDSCC (16/34, 47.1%) was also documented by Babiker et al.[20] but no association was found by Sritippho et al[18] who did not find any association of p16 with MDSCC. In case of poorly differentiated SCC, 14 out of 17 cases (82.3%), especially from oropharynx showed p16 while 11(64.7%) showed p53. No PDSCCS cases found in pyriform fossa. It is worth noting that there was significant association of histological grade with p16 (p <.001) but not with p53(p = 0.091) expression.

There were few limitations in this study. Firstly, number of patients were not equally distributed between different sites. Only patients from a single centre were included. Multicentre future research on type of HPV infection on pyriform fossa will through more lights on the prevalence of this infections. Inclusion of PCR based assays on tissues on different subtypes of HPV both high and low risks will yield better results.

CONCLUSION

It can be summarised that prevalence of HPV infection in SCCs of pyriform fossa though lesser than that of oropharynx ishighwhich proves that HPV-16 isassociated in their etiopathogenesis. Age does not differ much amongst patients of SCC at all sites of head and neck studied. Males are affected more as expected. Patients with both smoking and alcohol consumption expressed more p16. HPV positive SCCs tend to have morphology more akin to Moderated differentiated SCCs with or without evidence of keratinisation.

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