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Original Research Article

To study HPV and EBV in squamous cell carcinoma of upper aerodigestive tract by immunohistochemistry markers

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ABSTRACT

Background: Squamous cell carcinoma (SCC) in upper aerodigestive tract (UADT) is one of the predominant causes of mortality and morbidity in a developing nation like India. The major risk factors being tobacco usage and alcohol. Besides these there are certain viruses like high-risk Human Papilloma Virus (HPV) and Epstein Bar Virus (EBV). Which have a potential role in the etiology of SCC. More over these HPV positive tumors have better prognosis compared to HPV negative ones due their radiosensitivity. There are several methods employed in identification of these viruses of which IHC is cost effective, reliable with high sensitivity and specificity.

Methodology: In the present study, we identified 30 cases of SCC in UADT sent to Pathology department. Expression of p16 for HPV and LMP1 for EBV was performed on the tissue blocks manually.

Results: p16 was positive in around 17% (5/30 cases) whereas LMP1 showed 0% (0/30 cases) positivity. Majority of patients with p16 positivity in SCC of UADT are in the older age (above 50 years), showed moderately differentiated (Grade II) SCC, had nodal metastasis at the time of presentation.

Discussion and Conclusion: Some studies showed the association of HPV of SCC in UADT in younger population have better prognosis compared to HPV negative ones. In the current study HPV was identified (17%) in older population (above 55 years) and none of the cases showed positivity for LMP1(EBV).

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1. Introduction

Squamous cell carcinoma (SCC) in Upper aerodigestive tract (UADT) is broad subject. There are several risk factors for SCC, here we would like to study the association of HPV and EBV (Epstein Bar Virus). In a developing nation like India, the association of Human papilloma virus (HPV) in Oral Cancer (OC) might be an issue of controversy for two reasons – one, as the habit of abusing tobacco is much more common in India and the beliefs and culture of the country, where oral sex which are the main cause of HPV associated OC, are believed not to be much common. Even though the literature shows large data concerning HPV and OC, it is

difficult to say that in the absence of any other etiological factors, HPV can result in OC.^{1,2}

SCC associated with high-risk HPV (HPV 16, 18) has a better prognosis than HPV-negative. p16 IHC (Immunohistochemistry) has been recommended as a prognostic test because it is less expensive and time-consuming than other examinations.³

EBV can be detected even in people who have no symptoms, and it can last a lifetime as a latent infection. EBV has been investigated as a possible cause of a variety of epithelial cell cancers, including nasopharyngeal carcinoma (NPC). The frequency and consistency of EBV DNA found in oral squamous cell carcinoma (OSCC) suggest that EBV infection may play a role in OSCC development. Nevertheless, there were some discrepancies

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in the findings.^{4,5}

Latent membrane proteins (LMP) such as LMP1, LMP2A, and LMP2B are expressed in Nasopharyngeal carcinoma. Of these LMP1 specific membrane protein IHC antibody is used for identification of EBV.⁶

2. Aims and Objectives

2.1. Primary objective

To determine the presence or absence of HPV and EBV in SCC of UADT by IHC.

2.2. Secondary objective

To determine the association of HPV with EBV in SCC of UADT by IHC.

3. Materials and Methods

3.1. Study design

Cross-sectional study.

3.2. Inclusion criteria

All patients with biopsy confirmed UADT squamous cell carcinoma who underwent radical primary surgery (Figures 1, 2 and 3) including neck dissection.

3.3. Exclusion criteria

All small biopsies with inadequate material without TNM staging of SCC of UADT specimens were excluded from the study.

3.4. Study duration

October 2018- November, 2021.

3.5. Sampling technique

Consecutive sampling.

3.6. Sampling size

30.

3.7. Statistical method

Chi square test, Independent sample t test, Fisher exact t test.

3.8. Method of collection of data

A sample of 30 cases were taken with their clinical details and where adequate biopsy available for paraffin embedded tissue. All the clinical details were either collected from Laboratory information services and medical record section. The required sections were stained with Hematoxylin

and Eosin. Pathological grading of tumor was done by Broder's classification. Tumor, nodal, metastasis was used for pathological staging.

3.9. Procedure for p16 and LMP1 immunohistochemistry (IHC) staining

IHC was performed on 4 μ m thick sections on poly-l-lysine coated slides, deparaffinised with xylene and rehydrated in graded ethanol concentrations to distilled water.

Antigen retrieval was done with EDTA/ Tris buffer at pH 8.5 – 9 in a pressure cooker for 20 minutes. The sections are incubated with primary antibody p16 (DAKO, p16-INK4(MX007)- MAD-000690QD-R-3) and LMP1 (Epstein-Barr Virus /LMP1 (DAKO, CS1-4)-MAD- 001619QD-R-3) for 30 minutes followed by primary antibody amplifier for 15 minutes, then secondary antibody for another 30 minutes. This is followed by Diaminobenzidine as chromogen. Counter staining is done with Harris Hematoxylin, followed by clearing, mounting and labelling.

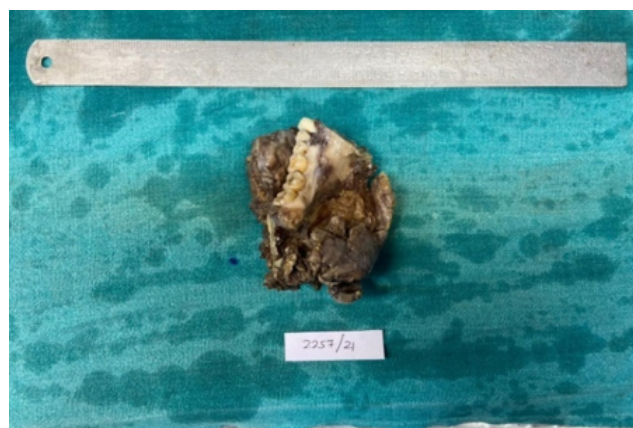


Fig. 1: Left mandibulectomy

Squamous cell carcinoma of cervix was taken as a positive control for p16 IHC. p16 IHC was considered positive if more than 70% of the tumor cells showing nuclear and cytoplasmic staining (Figures 4 and 5).^{7,8}

Classic Reed Sternberg cell in Hodgkin lymphoma was taken as a positive control for LMP1 marker. LMP1 was considered positive based on Allred scoring (Figures 9 and 10).^{9,10}

4. Results

4.1. Patient demographics

The age of the patients ranges from 40 years to 88 years with a mean age of around 60. Majority of the patients (16/30) were in the age group between 50-70 years accounting for more than 50% of the total cases (Table 1). Out of the 30 patients 21 (70%) were male and 9 (30%) were female. Male



Fig. 2: Laryngectomy



Fig. 3: Left segmental mandibulectomy

to female ratio was 2.3: 1 with higher prevalence seen in males (Table 2). There were patients (63.33%) with history of tobacco in form of either cigarette, beedi and chewable form (Table 3). Of the 19 cases with history of tobacco 13 were male and 6 were female. Among the non-tobacco users, 7 are male and 4 are female. Tumor is located in oral cavity in 19 (63%) cases, larynx 8 (26%) cases and hypopharynx 3 (10%) cases (Table 4). Majority of cases 25 (83%) were moderately differentiated SCC, followed by well differentiated 3 (10%) and poorly differentiated 2 (6.6%). Lymphovascular invasion was identified in 18

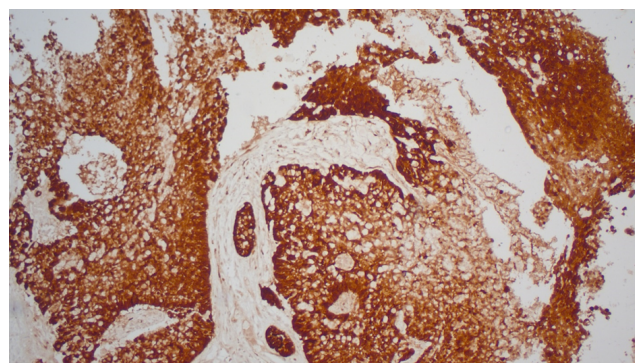


Fig. 4: p16 IHC control- Squamous cell carcinoma of cervix(10x), showing >90% cells positivity

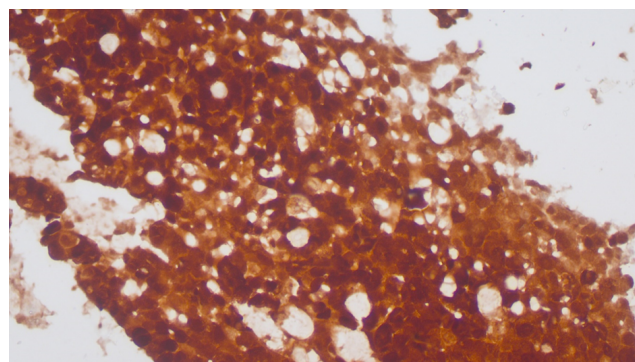


Fig. 5: Diffuse p16 positivity in >80% of cells, strong cytoplasmic and nuclear staining

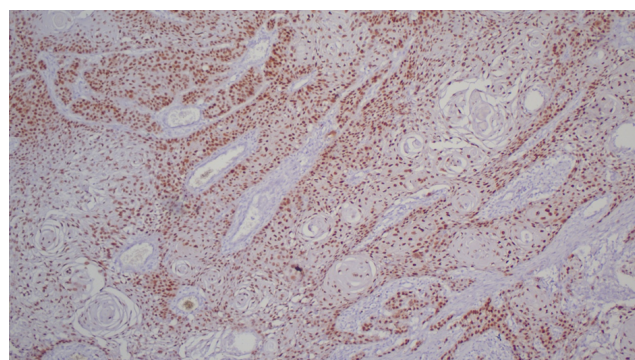


Fig. 6: Diffuse p16 positivity in >80% of cells, strong cytoplasmic and nuclear staining

(60%) cases and perineural invasion was identified in 8 (27%) cases. Majority of tumors presented at stage 3 (15/30 cases, 50%), followed by stage 2 (9/30 cases, 30%).

4.2. P16, LMP1 IHC correlation with clinicopathological variables

HPV p16 IHC positivity was seen in 5/30 (16.7%) cases (Figures 6, 7 and 8) and none showed positivity for EBV

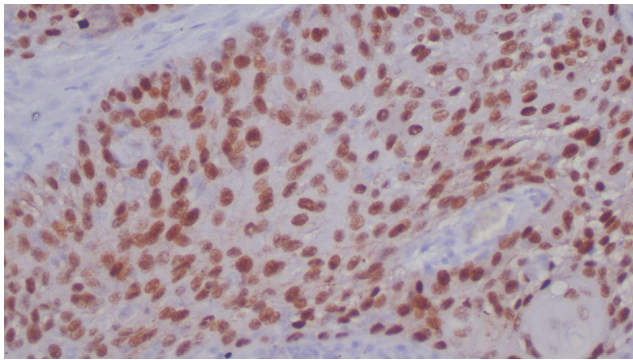


Fig. 7: p16 positive, strong nuclear positivity, moderate cytoplasmic positivity (400x magnification [40x objective])

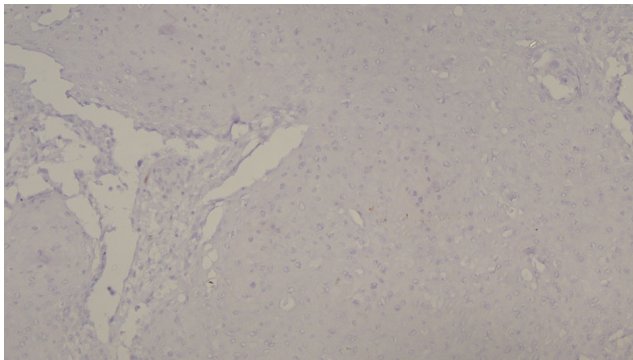


Fig. 8: p16 negative, no nuclear positivity and no cytoplasmic positivity (100x magnification [10x objective])

with LMP1 marker. p16 positivity was seen in elderly population (55-88 years) of which three were males and two were females. Three had history of tobacco and two didn't. Majority of p16 positive tumors were in oral cavity (3/5 cases, 60%), followed by 1 (20%) in larynx and 1 (20%) in hypopharynx. All the cases which showed p16 positivity were well differentiated SCC (5/5 cases, 100%). Lymphovascular invasion (LVI) was identified in 4/5 (80%) and perineural invasion (PNI) was seen in 2/5 (40%) cases in p16 IHC positive SCC. LMP1 marker for EBV was negative in all the 30 cases studied (Figure 11).

5. Discussion

Squamous cell carcinoma of the Upper aerodigestive tract has higher prevalence in males compared to females and majority of the cases are seen in 5th and 6th decade with a mean age of 60 years, which were correlating with other studies seen in Table 2 and Table 3. History of tobacco usage is present in majority of the cases and is major risk factor associated, which was correlating with other studies seen in Table 4. Tobacco history is mostly seen in male population. Presence of HPV by IHC is mostly seen in oropharyngeal region. In this study we evaluated 30 cases of SCC of UADT

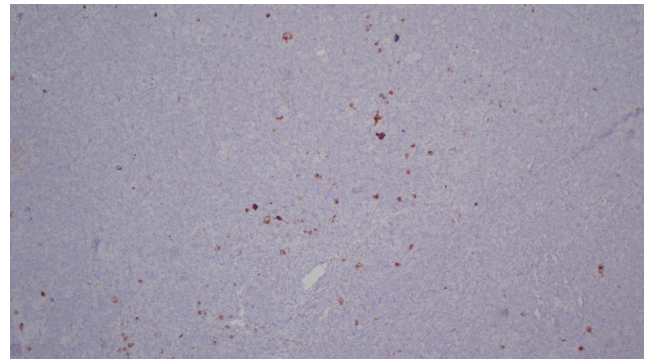


Fig. 9: LMP1 control (100x magnification [10x objective])

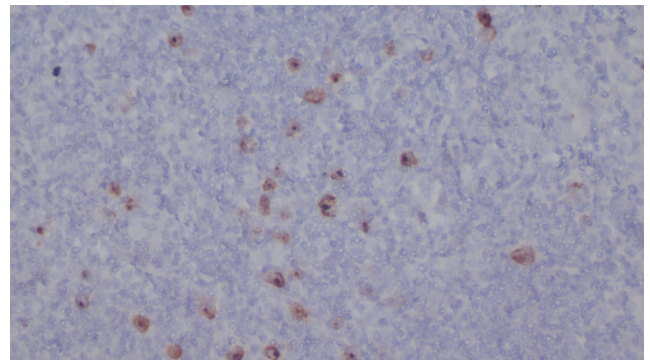


Fig. 10: LMP1 control (400x magnification [40x objective])

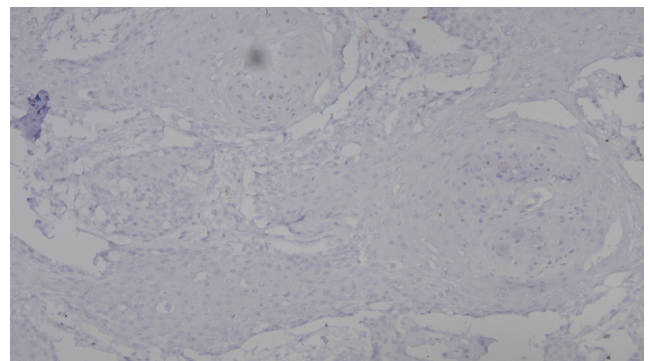


Fig. 11: LMP1 negative (10x)- in squamous cell carcinoma (100x magnification [10x objective])

and studied their clinicopathological variables along with p16 and LMP1 IHC markers.

5.1. Site of distribution of squamous cell carcinoma of UADT

Majority of tumors are found in oral cavity (63%) in the present study which is similar to other studies like Basu R et al, El-Mofty S et al and S Nair et al. Other studies (Table 5) like those of Dahlstrom et al, Cadoni et al and Riener M et al are discordant with our studies, showing

Table 1: Comparison of age

Study	Years	Age range (in years)
Basu R et al ¹¹	Oral cavity- 48 (Median), Larynx- 58 (Median)	30 - 75
Mahendra Pratap Singh et al ¹²	Male- 46.95, Female- 52.27 (Mean)	20 - 85
Dahlstrom et al ¹³	55.2 (Mean)	-
Schmidt Brain L et al ¹⁴	66.3 (Mean)	29 - 84
Verma et al ¹⁵	47.69 ± 6.73 (Mean)	-
Present study	60.03 (Mean)	40 - 88

Table 2: Comparison of male to female ratio

Study	Male to Female ratio
Rene Lambert et al ¹⁶	2.29 : 1
Vidaya R et al ¹⁷	3.74 : 1
S Nair et al ¹⁸	3.3 : 1
Dahlstrom et al ¹³	3.4 : 1
Cadoni G et al ¹⁹	4.3 : 1
Present study	2.33 : 1

Table 3: Comparison of tobacco usage

Study	Percentage of tobacco users
Basu R et al ¹¹	63.63%
Schmidt Brain L et al ¹⁴	67.00%
Subapriya et al ²⁰	66.7% (Chewers), 30.9% (Smokers)
Dahlstrom et al ¹³	67.40%
S Nair et al ¹⁸	69.3% (Chewers), 19.5% (Smokers)
Cadoni G et al ¹⁹	85.8%
Present Study	63.33%

Table 4: Comparison of tumor location

Study	Tumor Site			
	Oral cavity	Larynx	Pharynx	Others
S Nair et al ¹⁸	72.6%	7.7%	15.6%	Paranasal sinus and others 4.1%
Basu R et al ¹¹	55%	14%	-	Orofacial-25%, Thyroid-6%
Dahlstrom et al ¹³	19%	12.6%	68.4%	-
Cadoni G et al ¹⁹	17.2%	60.2%	21.6%	-
El-Mofty S et al ²¹	37.6%	-	-	Larynx/Hypopharynx- 25.8%, Oropharynx- 34.4%
Riener M et al ²²	28.2%	9.6%	62.2%	-
Present study	63%	26%	10%	-

tumors predominantly in oropharynx.

5.2. HPV presence by p16 IHC in Squamous cell carcinoma in UADT

In the present study 5 cases (5/30 cases, 16.66%) showed strong and diffuse (>70%) nuclear and cytoplasmic p16 positivity. There are few (3/30 cases, 10%) cases where there is a diffuse staining of nucleus and cytoplasm ranging from 20% - 30% with moderate to strong positivity, these

unequivocal cases are considered negative. The remainder of the cases were considered negative for p16 IHC where less than 20% percent of tumor cells are staining regardless of intensity.

Majority of the cases which are positive for p16 IHC are present in oral cavity (3/5 cases, 60%), one in pharynx (1/5 cases, 20%) and one in larynx (1/5 cases, 20%) which is consistent with study of Verma et al. Other studies (Table 5) like Riener M et al showed p16 IHC positivity predominantly in Pharynx (Oropharynx) followed by oral

Table 5: Comparison different studies with p16 IHC positivity and tumor location

Site		HPV p16 IHC positive				
		Lewis JS et al (n=239) ²³	Buajeeb W et al (n=16) ²⁴	Riener M et al (n=156) ²²	Verma et al (n=50) ¹⁵	Present study (n=30)
Oral cavity	Lip	-			0/3	0/1
	Buccal mucosa	-			0/12	2/9
	Tongue	-	3/16	16/44		
	Alveolus	-			4/16	0/4
	Palate	-			0/15	0/4
Larynx	-	-	6/15	-	1/8	
Oropharynx	187/239	-	59/97	-	-	
Hypopharynx	-	-	-	-	1/3	
Nasopharynx	-	-	-	2/2	-	

cavity.

HPV prevalence in OPSCC was highest in North America (65.8%) but higher in men than women and lowest in Asia (28.9%) where HPV prevalence in women was highest (61.5%) and higher in women than men. OPSCC has been found to have a disproportional rise in younger individuals under 45 years of age without significant cigarette or alcohol exposure. In this subpopulation females are more affected than males. Patients with HPV positive SCC were three times more often to report having had oral sex as those with HPV negative SCC. This subpopulation has a distinct clinical profile, with an earlier diagnosis, a higher risk of modest regional spread at the time of diagnosis, and a better prognosis and response to therapy.²⁵ Unlike other studies where HPV positivity was present in younger population below 50 years, in the current study we identified HPV positivity in much older population (above 55 years, 55, 61, 67, 70 and 88 years respectively). HPV-positive SCC were more likely to have an AJCC tumor stage of T3 & T4 than HPV-negative tumors (T2). (26) In the current study we identified patients with HPV positive SCC at higher, T3 stage.

According to few studies, patients with HPV-positive oropharyngeal cancer as detected by PCR, in situ hybridization, or P16 immunohistochemistry on tumor samples, have a considerably better overall and disease-free survival than those with HPV-negative oropharyngeal cancer.²⁵ When compared to HPV-negative cancer, HPV-positive oropharyngeal carcinoma is more sensitive to radiation and anticancer medicines, and has a better prognosis.²⁶

5.3. LMP1 IHC expression in SCC of UADT

There are several studies which showed the expression of LMP1 IHC for EBV in nasopharyngeal carcinoma, ranging from 0% in few studies to about 78%.^{10,27}

The presence of EBV was linked to neoplastic states of the oral cavity by Cruz I et al, but the information obtained does not suggest a direct involvement for EBV

in the malignant transformation of normal epithelial cells. It rather suggests that the appearance of EBV in OSCC samples is due to immune suppression leading to shedding of the virus in the oral cavity.²⁸

D Goldenberg et al, in their study observed that EBV was present in a small fraction of cases of head and neck tumors and they were detected at low levels, suggesting that the virus may not be contributing to tumorigenesis of head and neck SCC.²⁹

Here in our present study, the cases we got were from oral cavity, larynx and hypopharynx. All of these cases showed LMP1 IHC negative. The lack of positivity is due to lack of cases especially from the nasopharyngeal and oropharyngeal areas. Other reason might be clearing of the infection by the host immune system or failure of the IHC to identify the latent infection, where the viral DNA is integrated in host DNA silently, in such cases serum immunoglobulin, polymerase chain reaction (PCR) and Fluorescent in Situ Hybridization (FISH) could be useful in identification.

6. Conclusions

In this present study we studied the role of HPV and EBV association in squamous cell carcinoma of UADT. This study has identified the presence of HPV in 5 cases (17%), whereas none of the 30 cases showed positivity for LMP1 IHC. In the current study we identified HPV positivity in much older population (above 55 years). The cases which showed HPV positivity are of moderately differentiated, of these 3/5 (60%) had history of tobacco usage, 4/5 (80%) had LVI identified.

With the current literature showing HPV positive tumors having higher response rate to radiotherapy compared to HPV negative ones. This suggests the use of p16 IHC to identify the subgroup of SCC positive for p16 IHC and alter the treatment specific for HPV positive tumors by lowering the intensity of radiotherapy.

7. Recommendations

1. Cessation of tobacco and its related products.
2. Further studies need to be studied.

8. Limitation of the Study

1. Sample size was small because majority of the cases were referred to a cancer centre.
2. PCR/ FISH was not performed for confirmation in p16 IHC positivity for specific HPV types.
3. Follow up of the data was not available for prognosis and morbidity.
4. Sexual history was not available for majority of all the cases.
5. For EBV serum immunoglobulin studies are not performed to know previous exposure.

9. Source of Funding

None.

10. Conflict of Interest


The authors declare no conflict of interest.

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