ASSOCIATION OF HUMAN PAPILLOMA VIRUS IN ORAL SQUAMOUS CELL CARCINOMA

ABSTRACT

INTRODUCTION

Oral squamous cell carcinoma is the most common malignant epithelial neoplasm affecting the oral cavity.it is commonly seen in the lip, oral cavity, salivary glands. Worldwide, oral cancer accounts for 2%–4% of all cancer cases. Although tobacco and alcohol are the main etiologic factors in majority of these cancers, the etiological factor in 1/4th of the cases remains unidentified. There is a growing evidence that human papilloma virus (HPV) may act as a cocarcinogen along with tobacco which eventually results in oral cancers.

Hence, this study is focused on evaluating the expression of HPV in OSCC and to correlate the association of HPV in OSCC patients.

AIM

The aim of this study is to analyze the presence of human papilloma virus in oral squamous cell carcinoma using p16 tumor biomarker and to compare the presence of human papilloma virus in tobacoo related and non tobacoo related oral squamous cell carcinoma.

METHODOLOGY

A 100 histologically diagnosed OSCC patients were selected for the study and sections were made to evaluate the presence of HPV using P16 tumor marker.

RESULTS

Out of the total 100 patients the presence of HPV was seen in only 3 patients. Tongue was the common site with a male predilection. Even though non smokers were more in the study, the presence of HPV was seen in patients with smoking habit. Thus correlating the relationship between presence of HPV and smoking habit.

There is no significant association between OSCC and HPV in our study. Our study also proves that there is an association between HPV and smoking habit. However considering our sample size, a larger sample size can be consider to prove the association between OSCC and HPV.

KEY WORDS: Oral squamous cell carcinoma, HPV, p16, immunohistochemistry

1. INTRODUCTION

Cancer is one of the most common disease, which results in morbidity and mortality today, more than 10 million new cases and more than 6 million deaths occur each year worldwide. In India, oral squamous cell carcinoma (OSCC) is the most common malignancy accounting up to 40-50%[1]of all malignancies. Although tobacco and alcohol are the main etiologic factors in three-fourth of these cancers, the etiological factor in 1/4th of the cases remains unidentified Human papilloma virus (HPV) which mainly causes cancers of the cervix,anus and throat, some studies have shown some evidence that HPV could be carcinogen along with tobacco and might cause oral cancers.

Human papilloma virus is small, circular double stranded deoxyribonucleic acid (DNA) viruses that belong to the papillomaviridae family[2]. Over 130 HPV types are known; these are classified as low or high-risk, based on their association with cervical carcinoma. HPV-16 and 18 are the most commonly detected high-risk types HPV transforms infected epithelial cells and causes defects in genes controlling apoptosis, cell cycle, and DNA repair, thereby promoting tumor genesis[3].

The protein p16 is a cellular protein involved in cell cycle regulation. In normal cells, p16 protein is expressed in very low levels and is almost undetectable by IHC[4] .
Due to the transforming activity of E7 oncogene p16 is strongly expressed in tumor cells affected by HPV and may be easily detected by IHC. Hence, p16 positivity correlates strongly with HPV positivity. However, literature search revealed meager data exclusively correlating HPV and histological grades of OSCC[5].

Studies have postulated that patients with HPV associated cancers have better response to treatment and have better survival rates[4]. Hence, studies are required to understand the biology and prognosis rate of HPV related head and neck SCCSs especially in our Indian population where we have a significantly higher incidence of oral cancers.

Hence, this study is focused on evaluating the expression of HPV in OSCC using p16 tumor marker and to correlate the association of HPV in OSCC patients with smoking habit.

2.MATERIALS AND METHODS

This study was conducted in Department of oral and maxillofacial surgery,Yenepoya Dental college,Mangalore,Karnataka,India with a sample size of 100 patients who were clinically evaluated and histopathologically diagnosed and confirmed as OSCC.

IMMUNOHISTOCHEMISTRY TECHNIQUE(IHC)

- *Materials*

- Incubator (60℃)
- Stain less steel pressure cooker (2lt capacity)
- Hot plate / induction cook top
- Metal slide racks
- Staining rack or glass rods over a tray covered with dark paper
- Timer

- Tissue rolls
- Micropipette
- Chemical balance
- pH meter
- Ordinary refrigerator with freezer
- Slides with frosted ends
- Lead pencil
- Slide lables
- Glassware- beakers, measuring jars etc..
- Reagents
    - APS (3 Aminopropyl Triethoxysilane)
    - Acetone
    - Xylene
    - Absolute ethyl alcohol
    - 95% ethyl alcohol
    - Distilled water
    - Citric acid
    - NaOH crystals
    - Disodium hydrogen phosphate
    - Dihydrogen sodium phosphste
    - Sodium chloride
    - DPX

➢ *Procedure*

- Tissue processing
    - Formalin fixation ,routine processing, paraffin embedding.

- (A) Preparation of coated slides
    - Take clean and new slides and dip in plain acetone for 20-30 minutes.
    - Dilute 15 ml APS (3 aminopropyl triethoxysilane) in 1 liter of acetone.
    - Dip the slides in this solution for 5 miutes.
    - A quick dip of the slides in plain acetone.
    - Dry the slides at room temperature.
    - Once the slides are dry- store in the freezer.

(B) purchase poly-L-lysine coated slides

- Deparaffinisation
    - Slides are kept in ths incubator at 60℃ for 30 minutes.
    - Dip in xylene for 10 minutes.
    - Dip in xylene for 10 minutes again.
    - Dip in absolute alcohol/isopropyl alcohol- 5 minutes.
    - Dip in 95% alcohol/isopropyl alcohol-5 minutes.
    - Dip in distilled water- 2to 5 minutes (until the slides appears clean).

- Antigen retrival

- Prepare TRIS EDTA buffer at pH 9.
- Put buffer in pressure cooker.
- Allow the buffer to boil.
- When buffer boils keep the deparaffinised slides inside and close the lid of the pressure cooker.
- Close the cooker ,place the weight, and allow one whistle to occur.
- After one whistle-keep the cooker under running water to cool.
- Remove the weight-open the lid- remove the slides.
- Wash in distilled water-5 minutes.
- Immediately put in phosphate buffer-3 minutes.

- Staining procedure
  - Keep the slides on glass rods kept a tray covered with dark paper.
  - Peroxidise block-1 drop or to cover the section for 10 minutes.
  - Phosphate buffer wash- two changes , 3 minutes each.
  - Primary antibody-1 dropon the section for 45 minutes to 1 hour at room temperature.
  - Phosphate buffer wash- two changes, 3 minutes each.
  - Target binder : secondary antibody- 10 minutes each.
  - Phosphate buffer wash- two changes, 3 minutes each.
  - HRP enzyme for 15 minutes.
  - Phosphate buffer wash- two changes, 5 minutes each.
  - DAB working solution(freshly prepared) one drop on slide for 7 to 10 minutes.
  - Wash in distilled water- just a dip.
  - One drop mayer's heamtoxylin for 5 minutes.
  - Wash with distilled water.
  - Dehydrate , clear and mount with DPX.

3.RESULTS AND DISCUSSION

This Study was conducted on 100 patients who were clinically evaluated and histopathologically diagnosed and confirmed as OSCC. All the patients included in the study were divided based on the gender distribution, presence or absence of smoking, site and presence or absence of HPV.

TABLE 1 GENDER DISTRIBUTION

SEX	Frequency	Percent
F	34	34.0
M	66	66.0
Total	100	100.0

TABLE 1 SHOWS THE PRESENCE OF MALE AND FEMALE PATIENTS INVOLVED IN THE STUD. STATISTICAL ANALYSIS SHOWS MALE (66%) PREDILICTION AND FEMALE (34%) PATIENTS

TABLE 2 SITE OF THE LESION

	SITE	Frequency	Percent
	BUCCAL MUCOSA	38	38.0
	GBS	2	2.0
	LIP	6	6.0
	MAXILLA	1	1.0
	PALATE	3	3.0
	RMT	9	9.0
	TONGUE	41	41.0
	Total	100	100.0

TABLE 2 SHOWS THE SITE INVOLVED IN THE PATIENTS AND STATISTICAL ANALYSIS SHOWS TONGUE (41%) AS THE COMMON SITE INVOLVED AMONG THE PATIENTS IN THE STUDY

TABLE 3 PRESENCE OR ABSENCE OF HPV

HPV PRESENT/NOT	Frequency	Percent
NEGATIVE	97	97.0
POSITIVE	3	3.0
Total	100	100.0

TABLE 3 SHOWS THE PRESENCE OF HPV IN THE STUDY WERE STATISTICALLY THERE IS HPV NEGATIVE IN 97% AND POSITIVE IN 3% PATIENTS

TABLE 4 PRESENCE OR ABSENCE OF SMOKING

SMOKER/NON SMOKER	Frequency	Percent

Valid	NON SMOKER	57	57.0
	SMOKER	43	43.0
	Total	100	100.0

TABLE 4 SHOWS THE NUMBER OF SMOKER AND NON SMOKERS INVOLVED IN THE STUDY AND STATISTICAL ANALYSIS SHOWS PREDILICTION OF A PATIENTS WITH NON SMOKING HABIT (57%)

TABLE 5 : HPV PRESENT/NOT * GENDER

		GENDER		
		F	M	Total
HPV PRESENT/ABSENT	NEGATIVE	34	63	97
		35.1%	64.9%	100.0%
	POSITIVE	0	3	3
		.0%	100.0%	100.0%
Total		34	66	100

TABLE 5 SHOWS THE PRESENCE OF HPV POSITIVE AND NEGATIVE IN MALE AND FEMALE PATIENTS, STAISTICALLY MALE PREDILICTION IN HPV POSITIVE PATIENTS(3%)

TABLE 6 HPV PRESENT/NOT * SITE OF THE LESION

		SITE							
		BUCCAL MUCOSA	GBS	LIP	MAXILLA	PALATE	RMT	TONGUE	Total
HPV PRESENT/ NOT	NEGATIVE	37	2	6	1	3	9	39	97
		38.1%	2.1%	6.2%	1.0%	3.1%	9.3%	40.2%	100.0%
	POSITIVE	1	0	0	0	0	0	2	3
		33.3%	.0%	.0%	.0%	.0%	.0%	66.7%	100.0%
Total		38	2	6	1	3	9	41	100

Chi-Square Tests	Value	df	P-VALUE

Pearson Chi-Square	1.593[a]	1	.207
N of Valid Cases	100		

Chi-square test is used to find the association between the HPV and Gender.
Here, we observe that there is no significant association between the two.(may be bcoz of less sample in positive cases.)

TABLE 6 SHOWS THE COMMON SITE INVOLVED IN HPV POSITIVE AND NEGATIVE PATIENTS AND STATISTICAL ANALYSIS SHOWSC TONGUE AS THE COMMON SITIVE INVOLVE DIN HPV POSITIVE PATIENTS(39%)

Chi-Square Tests

	Value	Df	P-VALUE
Pearson Chi-Square	1.164[a]	6	.979
N of Valid Cases	100		

Chi-square test is used to find the association between the HPV and site.
Here, we observe that there is no significant association between the two.

TABLE 7 : HPV PRESENT/NOT * SMOKER/NON SMOKER

		SMOKER/NON SMOKER		
		NON SMOKER	SMOKER	Total
HPV PRESENT/NOT	NEGATIVE	57	40	97
		58.8%	41.2%	100.0%
	POSITIVE	0	3	3
		.0%	100.0%	100.0%
Total		57	43	100
		57.0%	43.0%	100.0%

TABLE 7 SHOWS THE PERCENTAGE OF SMOKERS AND NON SMOKERS IN HPV POSITIVE AND NEGATIVE PATIENTS, STAISTICALLY NON SMOKERS ARE MORE IN HPV POSITIVE PATIENTS(57%)

Chi-Square Tests	Value	df	P-VALUE
Pearson Chi-Square	4.100[a]	1	.043

N of Valid Cases	100		

Chi-square test is used to find the association between the HPV and use of tobacco.
Here, we observe that there is significantassociation between the two with p=0.043

Oral squamous cell carcinoma is the most common malignant epithelial neoplasm affecting the oral cavity. Worldwide, oral cancer accounts for 2%–4% of all cancer cases. The prevalence of oral cancer in india is around 45%[6]. It is estimated that more of 90% of all oral neoplasms are OSCC.

HPV have been detected in a variable proportion of HNSCC from 10% to 100%. This variation in the prevalence of HPV detection rate could be due to differences in anatomic locations of tumors or the techniques used in detecting HPV-DNA. In a Meta-analysis during (1980-1998) it was observed that the likelihood of detecting HPV in normal oral mucosa was 10.0% which was significantly less than that of OSCC which is 46.5%[7].

The present study was conducted from November 2016 to September 2018 to analyze the presence of human papilloma virus in oral squamous cell carcinoma using p16 tumor biomarker and to compare the significance of HPV in tobacoo related and non tobacoo related oral squamous cell carcinoma. The patients reporting to the department of Oral and Maxillofacial Surgery and Department of surgical oncology , Yenepoya university were included in the study.

Out of total 100 patients selected for the study 34 patients were females and 66 males. Our study shows significant association between gender distribution and OSCC since there is a male predilection in patients diagnosed with oral cancer. As reported by R Shenoy et al[8] that there is a significant association in the incidence of oral cancer among males which can be attributed to easy acceptance of habits by males. The consumption of tobacoo and other carcinogens as a mean of stimulants makes males more susceptible to OSCC.

This study shows that the patients diagnosed with OSCC, tongue as the most common site of incidence (41%) followed by buccal mucosa (38%), RMT(9%) and lip (6%). This correlates wiyth the study done by M.Selvamani et al in which he had found the tongue as the most common site of tumor(14.09%)[9]. Dhamthai et al says most of the oral cancer were encountered at tongue. The reason may be because of the carcinogens like tobacco, traditional pan and commercial pan etc, when they mix with saliva they have a tendency to pool at the bottom of the mouth and this site is covered by thin and non keratinized mucosa. As a consequence they provide less protection against the carcinogen[10].

In the present study out of the total 100 patients diagnosed with oral squamous cell carcinoma, only 3 patients shows the presence of HPV showing there is no association between OSCC and HPV. This correlates with the review study done by Aimee et al where they have reviewed 5046 SCC cases in which overall HPV prevalence was significantly higher in oropharyngeal SCC than oral cancer. The use of tobacoo related products, life style, culture remain the predominant case of HNSCC and HPV association especially in OSCC. 3% positivity of HPV in our study which was in accordance with Koppikar et al., Khovidhunkit et al., Zeuss et al. studies revealed 6%, 2%, and 0%, respectively exhibiting a low prevalence of HPV in OSCC[(11)].

While in the case of association between smoking habit and OSCC, 43% of patients were smokers and 57% of patients were non smokers but majority of the patients in this had a habit history like commercial pan and traditional pan chewing. In the study done by Nirmala et al has been proven that smoking in the form of bidi and cigarette causes high risk for oral cancer. The risk of developing oral cancer increase as the dose and the duration of smoking tobacoo increases.

In the patients with HPV positive male patients shows the predominance compared to females. A study done by Priya koppikar et al also found male predominance in their sudy and also in a study done by Werners et al also found statistical correlation with male predominance. This can be due to the increased rate of smoking and alcohol consumption among males.

In our study we have used p16 tumor biomarker using immunohistochemistry to detect HPV. Our study found 3% positivity of HPV which was in accordance with Koppikar et al., Khovidhunkit et al., Zeuss et al. studies revealed 6%, 2%, and 0%, respectively exhibiting a low prevalence of HPV in OSCC independent of the method used whereas Balaram et al., Kojima et al., Ostwald et al., who found 74%, 66%, and 62% HPV positivity, respectively[(12)] .

All the patients with HPV positive in our study has smoking habit which correlates with the study by sreejyothi et al showing the significant association between smoking habit and HPV in OSCC. Tobacoo have an addictive effect and alcohol consumption as a synergestic effect with HPV positive cancer. In these patients smoking while exposed to HPV can lead to retention of HPV in the oral cavity and oropharynx because of its local immune suppressing effects and develop HPV related OSCC later in life.

4.CONCLUSION

In our following study:

100 Patients who were evaluated and diagnosed histopathologically with oral squamous cell carcinoma for the correlation between OSCC and HPV and the relation between HPV and smoking habit.

Out of the total patients selected for the study 34 were females and 66 were males. While comparing the association between HPV and gender, our study showed that there is no significant association between the two.

Among the 100 OSCC patients only 3 patients showed the presence of HPV and the rest 97 were having absence of HPV showing no association between oral squamous cell carcinoma and human papiiloma virus.

Our study shows that among the patients diagnosed with oral squamous cell carcinoma, tongue was the most common site identified followed by buccal mucosa, RMT and lip. And there was statistically significant association between the site of the lesion and the presence of HPV since all the three patients with positive HPV was diagnosed with carcinoma of tongue.

Out of the total 100 patients selected for the study 43 were smokers and 57 were non smokers. While assessing the association between the HPV and use of tobacco, there was a statistically significant association.

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