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STUDY OF CORRELATION OF HUMAN PAPILLOMA VIRUS WITH ORAL CAVITY & OROPHARYNGEAL SQUAMOUS CELL CARCINOMA.

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ABSTRACT A prospective cohort clinical study was carried out at Dr.D.Y.Patil Hospital & Research Institute, Kohlapur from May 2014-May 2016. An attempt was made to correlate the Human Papilloma Virus in Oral Cavity and Oropharyngeal Squamous Cell Carcinoma. Real time Polymerase Chain Reaction technique using consensus HPV primers was used to detect HPV type 16 & HPV type18 in punch biopsy specimens from 70 patients of oral cavity & oropharyngeal squamous cell carcinoma. Data was collected from all patients by giving questionnaire and statistically analysed by applying chi-square test. 83.33% of HPV were detected in Oral cavity Squamous cell carcinoma & 16.66% of HPV were detected in Oropharyngeal Squamous cell carcinoma. Among HPV positive , majority 66.66% were HPV type 16 , 33.33% were HPV type16 & HPV type18 combined. HPV positive cases showed better prognosis & overall survival as compared to HPV negative cases. Thus we conclude that HPV positive cancers differ significantly from HPV negative cancers with regards to patient's age, gender, addiction, site of tumour and stage.

KEYWORDS : Oral Cavity Squamous Cell Carcinoma, Oropharyngeal Squamous Cell Carcinoma, Human Papilloma Virus .

INTRODUCTION

Head and neck cancer encompasses epithelial malignancies that arise in the paranasal sinuses, nasal cavity, oral cavity (including the buccal mucosa, upper and lower alveolar ridges, retromolar trigone, floor of the mouth, hard and soft palate and anterior two thirds of the tongue), oropharynx, nasopharynx, hypopharynx, pharynx, and larynx[1]. The incidence and mortality of head and neck cancer varies throughout the world. Globally, it is the sixth most common cancer type, accounting for 6 % of all cases, and responsible for 650 000 new cancer cases annually with a mortality of 350 000 cases annually worldwide[2]. The Indian sub-continent accounts for one-third of the world burden. The incidence and mortality from oral cancer is rising in several regions of Europe, Taiwan, Japan and Australia. About 90 percent of head and neck cancers are of the squamous cell variety.

Oral cancer is the most common cancer in India; as 4 in 10 of all cancers are oral cancers. Annually 130,000 people succumb to oral cancer in India which translates into approximately 14 deaths per hour. The reason for high prevalence of oral cancer in India is primarily because tobacco is consumed in the form of gutka, quid, snuff or misri. Rising tobacco use in India, where 40 per cent of the world's smokers live has contributed to this trend. . In India, 20 per 100000 population are affected by oral cancer which accounts for about 30% of all types of cancer[3]. Over 5 people in India die every hour everyday because of oral cancer[4].In India, cancer of the oral cavity and oropharynx is the commonest cancer in men and third commonest cancer in women [5].Oral cancers are more common in males than females. However, there is a rise in the incidence of these malignancies in females. Oral cavity cancer incidence in males is 50,174 (10.9%) and in female is 28,245(0.53%) whereas oropharyngeal cancer incidence in males is 39,098(0.5%) and in females 13,132 (0.16%) [6]. Incidence of oral cancers in patients with risk factors is 8.4 times higher than that of patients who do not have any risk factors and male to female ratio is 3:1. This ratio is seen in most of the published studies in India. Oral cancers implies quite significant mortality and morbidity rates and in spite of the vast amount of research and the advances accomplished in the field of oncology and surgery, the mortality rates remain unchanged. This motivates the search of factors with prognostic relevance in order to better tailor the individual management of OSCC patients. The purpose of this article is to list and discuss various factors, mainly focusing on HPV correlation with Oral & Oropharyngeal cancers.

Epidemiology : Despite increased understanding of oral cancer carcinogenesis, fully supported by the most recent technology including combined treatment modalities of oral cancer management, diagnosis has not improved significantly with regard to the 5 year overall survival rates for the disease over the past three decades especially in the United Kingdom (UK)[7][8]. Based on global incidence, the group of oral and oropharyngeal cancers rank sixth among human cancers[8]. Geographic variations in oral cancer incidence are greatly influenced by factors such as genetic, habits and lifestyle[9]. High-risk countries for oral cancer are India, Pakistan, Sri Lanka and Bangladesh. About 95% of all oral cancer cases occur in people over the age of 50 and twice as frequently in men as in women. Nowadays, a new trend of oral cancer in young people before the age of 40 is emerging and the male:female ratio has been changing significantly, from 5:1 to 2:1, over the last five decades. In Asian populations, the buccal mucosa is the most common intraoral site for mouth cancer, whereas tongue is the commonest among European and US cases[8] .

Risk factors for oral cancer:

It is widely considered that the major risk factors for oral cancer are tobacco smoke [10] [11], alcoholic beverage drinking [10] or synergy between the former and later factors [12].

Tobacco smoking: More than 300 carcinogens are present in either tobacco smoke or its water-soluble components in heavy smokers' saliva [11]. The first 69 carcinogens were previously reported and categorised into eleven groups. It was

observed that dose-related tobacco smoking practice (i.e. the frequency of cigarettes smoking and duration of smoking) is strongly associated with oral cancer[13][14][15].

Smokeless tobacco: Smokeless tobacco is an alternative way of consuming tobacco, whereby combustion is not required. Nevertheless, nasal snuff in various forms (loose or small packet), and chewing tobacco (in block or flakes) still keeps mucous membranes in contact with the carcinogenic agents[16].

Among the other factors, Betel quid or arecanut chewing, alcohol & dietary factors have been associated as a major risk factors for Head & Neck squamous cell carcinoma [17][18][19][20].

HPV as risk factor

Over the last 10-15 years HPV infection has been increasingly recognized as a major aetiological factor for HNSCCs[21][22][23][24] ,mostly OPSCC. HPV infection in the aetiology of OPSCC was first shown by Gillison et al.[25], numerous case series and studies conducted in the late 1990s and early 2000s evaluated the prevalence HPV infection in oropharyngeal cancer using molecular techniques such as PCR and in situ hybridization[22][26][27]. Over the last five years it has become increasingly clear that HPV plays a pathogenic role in this subset of head and neck cancers, with distinct epidemiologic, clinical and molecular characteristics. These findings have created new opportunities for improved therapy and primary prevention for these HNSCCs[28].

HPV is a DNA oncovirus and is epitheliotropic. Approximately 200 HPV genotypes belonging to 49 species have been recognized by the International HPV Reference Centre. These genotypes have been sequenced and classified according to their phylogenetic position, biological niche and oncogenic potential with new types discovered regularly. The genera alpha, beta, gamma, mu and nu types infect the humans. Novel -HPV types are believed to be present in oral cavity of healthy individuals also. Most recently, a novel HPV type 199 (HPV199) was Identified in a nasopharyngeal swab sample[29]. There are 30–40 genotypes from the -genus of HPVs that infect the human genital tract. Based on their oncogenic potential, they can be subdivided into low- and high-risk types. Low-risk HPV types include HPV6 and 11 which have been associated with benign warts or condylomata. By contrast, there is at least 12 high risk HPV types, HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59. They have been associated with at least six different cancers as well as precursor neoplastic lesions. HPV 16 and 18 are well-established initiators of 90% of cervical cancers, 70% of anogenital cancers, 5% of non-oropharyngeal SCC[30] and 20-72% of OPSCC [30][31][32].

MATERIALS & METHODS:

A Prospective cohort clinical study was carried out at Dr.D.Y.Patil Hospital & Research institute, Kolhapur(MAHARASHTRA), from May 2014 to May 2017,after obtaining approval from the ethical committee of the institute. All patients attending the E.N.T., outpatient department, with oral lesions, were screened & examined. Punch Biopsy was taken & sent for the histopathological report. The patients with positive for Squamous Cell Carcinoma were included in the study. The paraffin embedded blocks were prepared & sent for HPV DNA detection. HPV DNA detection & genotyping of these blocks was performed by pathologist. The WHO grading system was used for the histopathological grading of oral cavity and oropharyngeal squamous cell carcinomas. This system is largely based on the Broders classification and uses three grades Grade I (Well differentiated), Grade II (Moderately differentiated) and Grade III (Poorly differentiated)[33]. The staging of oral cavity and oropharyngeal cancers was based on the TNM system of the American Joint Committee on Cancer (AJCC)[34].On the basis of stage at presentation the patients were divided into two groups: those with early stage disease (Stage I/II) and those with late stage disease (Stage III/IV).

Prevalence of HPV infection in oral and oropharyngeal malignancies was evaluated by Human Papilloma Viruse types 16 & 18 Fluorescent Real Time Polymerase Chain Reaction Diagnostic test. Chi-square test was employed to test the association of different variables with HPV status and a p value of more than 0.05 was taken as insignificant.

- **PCR TECHNIQUE:** Genomic DNA was isolated from the biopsy tissue by phenol–chloroform extraction method[35]. PCR was carried out with the HPV consensus primers MY09/11.The details of primers are as follows:
- MY09 5 —CGT CCA AAA GGA AAC TGA Tc—3 .
- MY11 5 —GCA CAG GGA CAT AAC AAT Gg—3 .
- GP5+ 5 — TTT GTT ACT GTG GTA GAT ACT Ac—3 .
- GP6+ 5 — GAA AAA TAA ACT GTA AAT CAT ATT C —3 .

MY(consensus primer),GP(general primer) and TS (type-specific primer). Initial denaturation step at 95°C for 5 minutes, followed by 40 cycles of denaturation at 55°C for 1 minute , annealing at 55°C for 1 minute and extension at 72°C for 10 minutes.

Sample positive for general primer, were further evaluated for HPV genotype 16 & 18 using type specific primer (TS 16 & Ts18). PCR was performed using 300ng DNA as template. Results were analysed on 1.5% agarose gel containing 0.5μg/ml ethidium The products were electophoresed on 1.5% agarose gel and stained with ethidium bromide. A band at 450 base pair specific for HPV was seen. Lane N: negative control, Lane P : positive control, Lanes 1-4 HPV (general primer) positive tumuor samples , Lanes 5-6 HPV 16 positive tumuor samples , Lanes 7-8 HPV 18 positive tumuor samples, Lane L: molecular size marker (450bp ladder marker) Figure1.

RESULTS:

Of the 70 cases of oral cavity and oropharyngeal malignancies, 61(87.14%) were males and 9(12.87%) were females (Figure2). Mean age at diagnosis was 53.28 $\pm$ 11.21year, where majority 52.85% patients were in age group of 41to 60 years, followed by 28.57% in more than 60 years and only 18.57% were in 21 to 40 years of age group (Figure 3).

It was found that 64(91.42%) patients were having addiction in different forms and 6(8.57%) were not (Figure 4). Among Non addiction group, all were positive for HPV whereas in addiction group, all were negative for HPV.

It was observed that 57(81.42%) patients had malignancy of oral cavity and 13(18.57%) had malignancy of oropharynx. In the HPV positive group, 5(83.33%) patients had malignancy of oral cavity and 1(16.66%) had malignancy of oropharynx. Whereas among HPV negative patients 52(81.25%) had malignancy of oral cavity and 12(18.75%) had malignancy of oropharynx (Figure 5, 7).

Among the 70 patients, 2(2.85%) patients had stage I malignancy, 30(42.85%) had stage II, 10(14.28%) had stage III and 28(40%) had stage IV malignancy. 1(1.42%) of our patient in stage I underwent surgery with neck dissection , 1(1.42%) patient underwent surgery without neck dissection , all were subjected to post- operative radiotherapy.18(25.71%) of our patients in stage II underwent surgery with neck dissection ,

12(17.14%) patients underwent surgery without neck dissection ,29(41.42%) were subjected to postoperative radiotherapy and only one(1.42%) underwent postoperative chemotherapy. All of our patients In stage III underwent surgery with neck dissection & were subjected to postoperative chemotherapy. 4(20%) of our patients in stage IV underwent surgery with neck dissection, 14(20%) underwent surgery without neck dissection & majority were subjected to postoperative chemotherapy and only one was subjected to postoperative radiotherapy(Table 1).

It was observed that in a total 70 patients of oral cavity and oropharyngeal carcinomas 6 (8.57%) were found to be HPV positive and 64(91.42%) were found to be HPV negative by PCR technique(Figure 6). Among HPV positive , majority 4 (66.66%) were HPV type 16 , 2(33.33%) were HPV type16 & HPV type18 combined.

Among the 70 patients it was observed that 39 (55.71%) patients had well differentiated malignancy, 25 (35.71%) had moderately differentiated malignancy and 6 had poorly differentiated malignancy. In HPV positive cases, 6(8.57%) all were poorly differentiated. However in HPV negative cases, 39(55.71%) were well differentiated, 25(35.71%) were moderately differentiated and no one was poorly differentiated(Figure8).

In present study, 6 (8.57 %) were detected as HPV positive , out of which 83.33% were disease free , 16.67% were lost to follow up , whereas 64 (91.42%) were HPV negative , out of which 82.81% were disease free , 6.25% were lost to follow up & 7.83 % were expired (Table 2).HPV positive cases showed better prognosis and overall survival rate as compared to HPV negative cases. As p value <0.001 i.e. p value <0.05 which shows significance (Figure 9).

DISCUSSION:

India is a high-risk region for oral and oropharyngeal cancers due to a high prevalence of tobacco use, particularly chewing (in both sexes), bidi smoking and alcohol drinking in male population. Tobacco and alcohol are the two most important known risk factors for the development of oral cancer. Cofactors in oral squamous cell carcinoma include dietary factors, immunodeficiency and viral infections like HPV 16/18. PCR study for HPV was done in 70 patients out of which 8.57% were positive & rest were negative. The same was true in other studies (Prashad et al[36], M.L Gilision et al[37], Tanu Agrawal et al[38], Akhter M et al[39], Ashutosh et al[40]).

The reported rates of detection of HPV DNA in oral cavity & oropharyngeal squamous cell carcinoma in various studies range from 0% to 100%. The extreme variation in reported prevalence may be owing to lumping together of essentially different lesions, to different sample numbers ranging from 2 to 100 samples and to difference in sampling techniques(use of frozen or paraffin embedded tissue sample).In the ethno-geographic origins of the subjects examined and in the HPV detection methods applied. Considering the participants understanding of the definition of oral sex, one should be cautious in interpreting these figures.

The range of age group in our study was 18 years & above. Mean age was 53.28 $\pm$ 11.21years. The minimum age of the patients in our study was 28 and maximum was 75 years. Maximum numbers of patients were in the age group between 41 to 60 years. The same was noted in other studies(Saghravanian N et al[41], Saklain MA et al[42] ,J S Polling et al[43]). Oral HPV infection is considered to be bimodal in age distribution, with a high prevalence in younger (30-50 years of age) & in later years (60-64 years). The patients who were HPV+ve in our study were between 46 and 70 years of age. It has been speculated that the higher HPV prevalence at younger age could be due to different sexual behavior, whereas at older ages could be due to increased duration of infections at older age, rather than increased acquisition of new HPV infections.

In present study it was seen that males comprised 87.14% and females comprised 12.87% showing male preponderance. This finding was similar to the study by Saghravanian N et et al[41] ,M.L Gilision et al[37].

The history of tobacco and alcohol use was found to be less common in HPV positive patients than HPV negative patients. Study by CL Davidson et al[44], showed that 80% were drinkers, 46.4% were smokers , 40.8% were practiced oral sex. Study by Ashutosh et al [102], showed that 37% were habituated to combined addiction, 27.5% were drinkers, & 0.3% were practiced oral sex. In western studies oral sex was common risk factor, but in our study none of the patient gave history of oral sex. The reason for this may be social enigma. In western countries, oral sex is practiced commonly but in india this is not.In our study there were some patients who didn't have any addiction, but were positive for HPV. Probably reason for this may be that they were practicing oral sex but did not commit.
In the present study, the most frequent subsites of involvement in carcinoma of oral cavity were buccal mucosa (60%) & in carcinoma of oropharynx were base of tongue (17.14%). The same was true in other studies.(Smith et al[45] Sanjeev Parshad et al [36] , Ashutosh et al [40] , Kristina R. Dahlstrom et al [46] , Daniel Brandizzi et al[47].Buccal mucosa is most common site for oral cavity malignancy in India. This is chiefly attributable to the common habit of placing tobacco & betel quid in gingival sulcus. In oropharynx, base of tongue is most common site in our study & same was reported in western countries because of smoking.

In our study , majority (42.85%) had stage II lesions, whereas in other studies (Sannigrahi et al[48], Smith et al[45]) more number of cases were found to be in stage III & stage IV. When diagnosed at early stage, prognosis & overall survival rate were better.

The histopathological grading of oral & oropharyngeal malignancy cases in the present study showed that among HPV positive patients all were poorly differentiated SCC 6(100%) , whereas among HPV negative patients, 39(60%) were WDSCC followed by 25(39.06%) were MDSCC and none were PDSCC. According to review of literature in published studies, HPV positive cancers have been recognized as a separate entity. They have different histologic features, do not undergo significant keratinization & are usually poorly differentiated. The same was true in our study.

In our study , among HPV positive , majority 66.66% were HPV type 16 , 33.33% were HPV type16 & HPV type18 combined. Studies by Herrero RC et al[49] , Balaram et al[50] ,Nagpal JK et al[51], Dcosta J et al[52], they had detected other HPV genotypes along with HPV type16 & HPV type18. In our study we had detected HPV only for High risk types (HPV type16 & HPV type 18) which are most commonly associated with oral cancers. We studied only two genotypes HPVtype16 & HPV type18 to keep the study cost effective.

In present study, most of the patients were subjected to postoperative chemotherapy in stageIV 27(38.57%), postoperative radiotherapy in stageII 29(41%) & some to chemotherapy. Whereas in a study by Klaus DW et al [53] postoperative radiotherapy or radiochemotherapy were given in advanced stage. According to literature , HPV positive cancers give better response to radiotherapy.

In our study follow up period ranged from 3moths to 1 year . Among the 70 patients 15(27.27%) had mucositis,13(23.63%)

had flap necrosis, 10(18.18%) showed pigmentation, 3(5.45%) had recurrence of disease , 1(1.81%) had xerostomia, 1(1.81%) had swelling, 1(1.81%) had trismus and & 1(1.81%) had dysphagia. Study by Astrid L Kruse et al[54] , during his follow up period of 1 year ,among the 67 patients 22.38% had recurrence of disease , 14.92% had distant metastasis and 4.47% had distant metastasis & recurrence of disease combined. In a study by Saghravanian N et al[41], 30.30% had recurrence of disease, 2% had distant metastasis & 1% had second primary cancer. In present study, most of the patients had radiation mucositis &only 5.45% had recurrence of disease who took inadequate postoperative radiotherapy. None of the patients showed distant metastasis.

In our study , HPV positive cases showed better prognosis & overall survival rate. As p value <0.001 i.e. p value <0.05 which shows significance.It is well established that HPV positive cases have better prognosis as compared to HPV negative cases.

In our study , 83.33% of HPV were detected in oral cavity Squamous cell carcinoma and only 16.66% of HPV were detected in Oropharyngeal Squamous cell carcinoma, whereas in other studies (M.L Gilision et al [37], Tanu Agrawal et al [38]) most of HPV cases were detected in oropharyngeal malignancy, but in our study only 16.66% of HPV were detected in oropharyngeal malignancy in whom who had no addiction.

In our study, association of HPV in oral cavity & oropharyngeal malignancy was insignificant (p value >0.05) by chi-square test (Figure10).

In conclusion, HPV positive cancers differ significantly from HPV negative cancers with regards to patient's age, gender, addiction, site of tumuor & stage. However, more Indian studies are required before HPV may be recognized as tumuorogenic factor in Oral cancers.

DECLARATIONS
Funding: None
Conflict of Interest: None
Ethical Approval: Not Required

Figure1. PCR amplification of HPV general, HPV type 16 and HPV type 18

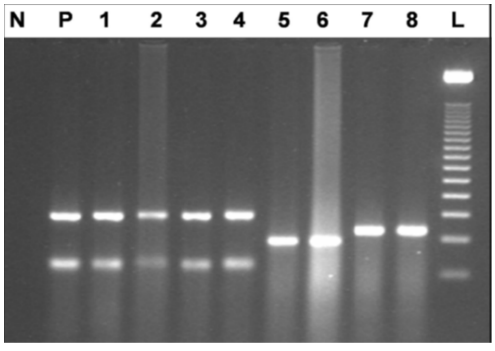


Figure 2. Gender wise Distribution

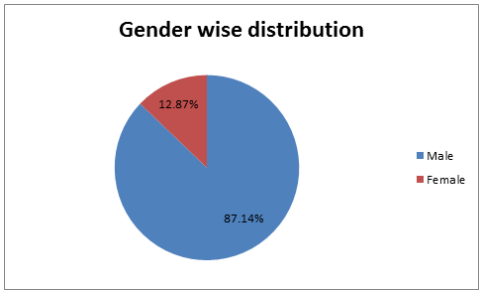


Figure 3. Age wise distribution

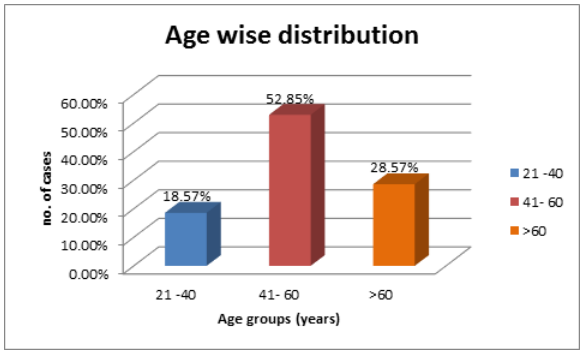


Figure 4. Distribution of Addiction type

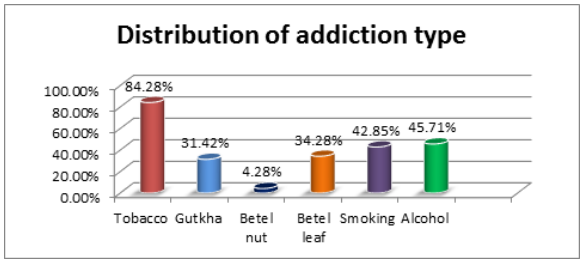
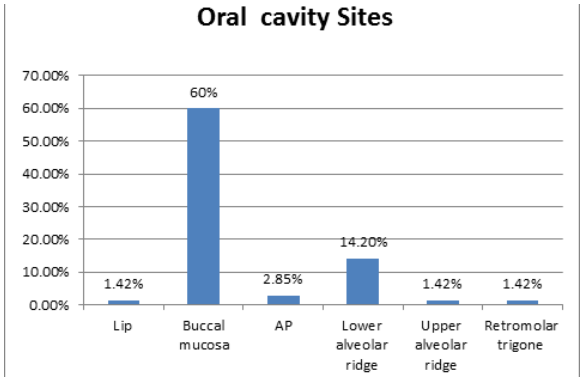


Figure 5. Distribution of sites among oral cavity and oropharynx



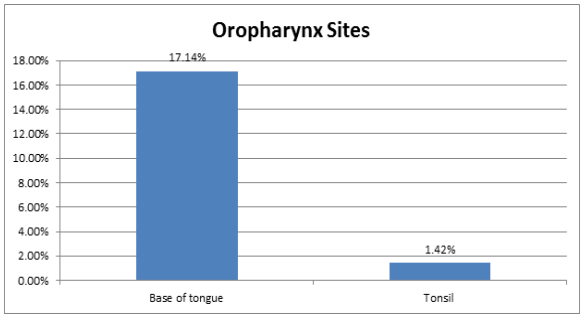


Figure 6: HPV detection by Polymerase Chain Reaction

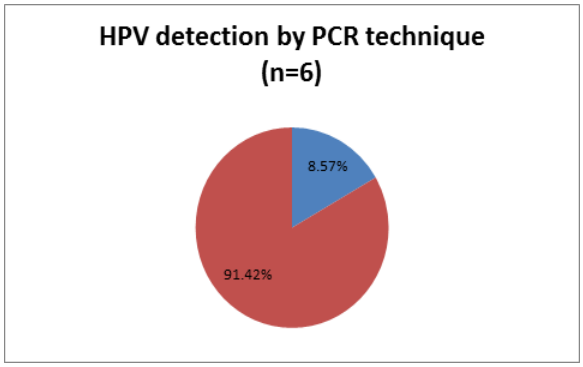


Figure 7: Presence Of HPV in Oral Cavity & Oropharynx

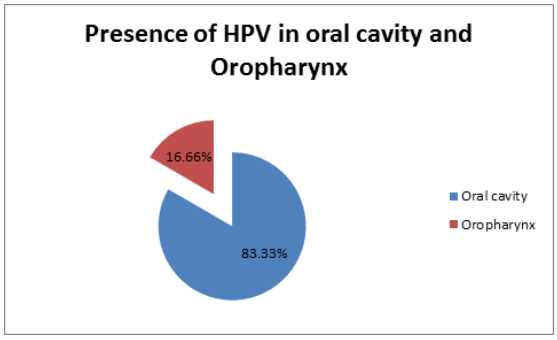


Figure 8: Histological Grading & its association with HPV presence

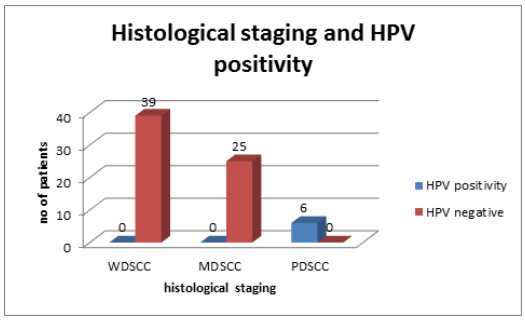


Table 1. TNM staging & treatment given in different stages

TNM staging	No. of patients	Percentage
Stage I	2	2.85%
Stage II	30	42.85%
Stage III	10	14.28%
Stage IV a	28	40%

Stages	Oral cavity/ Oropharynx (%)	Surgery (%)	With neck dissection (%)	Without neck dissection (%)	Chemotherapy (%)	Radiotherapy (%)
Stage I	2(2.84)	2(2.84)	1(1.42)	1(1.42)	0(0)	2(2.85)
Stage II	30(42.85)	30(42.85)	18(25.71)	12(17.14)	1(1.42)	29(41.42)
Stage III	10(14.28)	10(14.28)	10(14.28)	0(0)	10(14.28)	0(0)
Stage IV	28(40)	28(40)	14(20)	14(20)	27(38.57)	1(1.42)

Table 2: Follow up done to the patients

Parameter	No of patients	Percentage
Recurrence	3	5.45%
Flap necrosis	13	23.63%
Pigmentation	10	18.18%
Mucositis	15	27.27%
Xerostomia	1	1.81%
Swelling	1	1.81%
Trismus	1	1.81%
Dysphagia	1	1.81%

Figure 9 : Prognosis

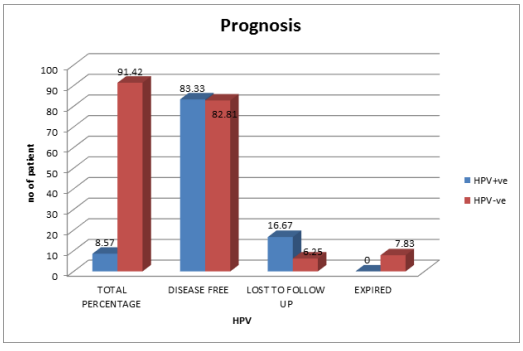
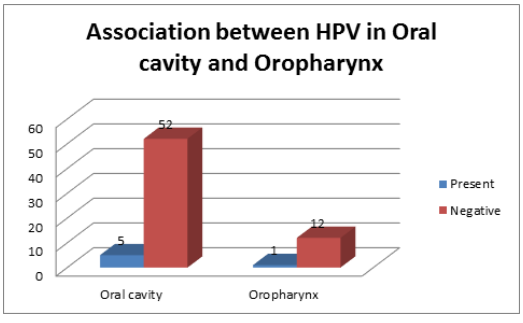


Figure 10: Association of HPV in Oral cavity & Oropharynx



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