Journal Homepage: -www.journalijar.com

INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR)

Article DOI: 10.21474/IJAR01/15198
DOI URL: http://dx.doi.org/10.21474/IJAR01/15198

RESEARCH ARTICLE

QUANTITATIVE AND QUALITATIVE ANALYSIS OF P16 IN HPV POSITIVE AND HPV NEGATIVE ORAL SQUAMOUS CELL CARCINOMA- AN IMMUNOHISTOCHEMICAL STUDY

Dr. Panchali Bora[1], Dr. Sanjeet Singh[2], Dr. Kanika Sharma[3], Dr. Nishant Singh[4] and Dr. Paramjit Singh[4]

1. MDS 3 rd year student, Dept of Oral and Maxillofacial Pathology, D.J College of Dental Sciences and Research, Modinagar, Distt.Gaziabad (UP).
2. Professor and Head of the Department,Dept of Oral and Maxillofacial Pathology, D.J College of Dental Sciences and Research, Modinagar,Distt.Gaziabad (UP).
3. Reader, Dept of Oral and Maxillofacial Pathology, D.J College of Dental Sciences and Research,Modinagar ,Distt.Gaziabad (UP).
4. Professor, Dept of Oral and Maxillofacial Pathology, D.J College of Dental Sciences and Research , Modinagar,Distt.Gaziabad (UP).

Manuscript Info

Manuscript History
Received: 07 June 2022
Final Accepted: 14 July 2022
Published: August 2022

Key words:-
HPV, Oral Cancer, $p16^{INK}$, Immunohistochemistry

Abstract

Diagnosing and prognosis of oral cancer precursors is challenging. This study investigates the correlation quantitative and qualitative expression of p16 in HPV positive and HPV negative OSCC cases .Human Papillomavirus (HPV) is believed to promote the oncogenic process and the correlation between viral oncoproteins and dysfunction of p16 tumour suppressor protein in oral lesions has been established in many studies .Concerning involvement of risk factors ,clinical course of the disease ,and prognosis there are strong indications arguing that the HPV positive OSCC may represent a separate tumour entity. Looking for a surrogate marker ,which in further epidemiological studies could replace the laborious and expensive HPV detection methods this study was conducted which include 30 cases of HPV positive and 30 HPV negative OSCC and 10 controlled cases of HPV colon were taken and p16 was evaluated according to distribution extent and degree of intensity.Based on the staining intensity among the HPV negative cases the staining intensity score was 1 in 80 percent of the subjects whereas in the HPV positive cases 63.3% were having Score 3 and 26.7% were having Score 2. The difference between HPV+ and HPV- was statistically significant and was analyzed using chi square test.Based on the semi quantitative analysis among the HPV negative cases the staining was negative in 80 percent of the subjects whereas in the HPV+ 63.3% were having diffuse staining and 30.0% were having focal staining. The difference between HPV positive and HPV negative was statistically significant when analyzed using chi square test.These data indicated that p16 is technically simple immunohistological marker applicable for routine pathological histology.

Corresponding Author:- Dr. Panchali Bora
Address**:-** MDS 3 rd year student, Dept of Oral and Maxillofacial Pathology, D.J College of Dental Sciences and Research, Modinagar, Distt.Gaziabad (UP).

Introduction:-

Cancer is defined as a group of disease involving abnormal cell growth with the potential to invade or spread to other parts of the body. It is one of the most common cause of death after coronary heart disease, causes nearly 7 million deaths each year worldwide. Presently almost 25 million people are suffering from cancer and by 2021 and it is projected that there may be 16 million new cases and 10 million cancer cases. It is marked that 6^{th} and 7^{th} decade of age group.

Head and neck cancer is not a specific entity, but rather a broad category of diverse tumor types arising from various anatomic structures including the craniofacial bones, soft tissues, salivary glands, skin, and mucosal membranes. The vast majority (more than 90%) are Head and Neck Squamous Cell Carcinomas (HNSCCs). The term head and neck cancer is often used to describe all carcinomas arising from the epithelium lining the sinonasal tract, oral cavity, pharynx and larynx and showing microscopic evidence of squamous differentiation.Various etiological factors involved in HNSCC can be categorized as chemical, biological and other environmental entities, which are dietary factors, socioeconomic status, tobacco use, alcohol consumption, viral infections like HIV, oral hygiene status, sharp tooth, family history, heavy metals, metabolic disorders like diabetesmellitus etc which results in uncontrolled and unorganised proliferation of cells by the involved carcinogen. HPV detection methods, such as morphology, in situ hybridization, Southern blot etc are technique sensitive and lack sensitivity and specificity, as well as the ability to detect high-risk HPV types. Therefore p16 immunohistochemistry was considered the most appropriate method.The tumour-suppressor protein p16 (also known asnp16INK4a, cyclin-dependent kinase inhibitor 2A) inhibits thebinding of the cyclin-dependent kinases 4 and 6 to cyclinD1. This inhibits the phosphorylation of retinoblastomaprotein (RB), which is needed for release of the E2Ftranscription factor to enable E2F to enter the cell cycle. This pathway can be disturbed by a -HPV infection by the viral oncoprotein E7. Thus, p16 can serve as a surrogate marker for -HPV infection and is used both in clinical and scientific protocols.

Aims And Objectives:-

To evaluate the expression of p16 biomarker in patients of Oral Squamous Cell Carcinoma associated with or without the presence of Human Papilloma Virus.

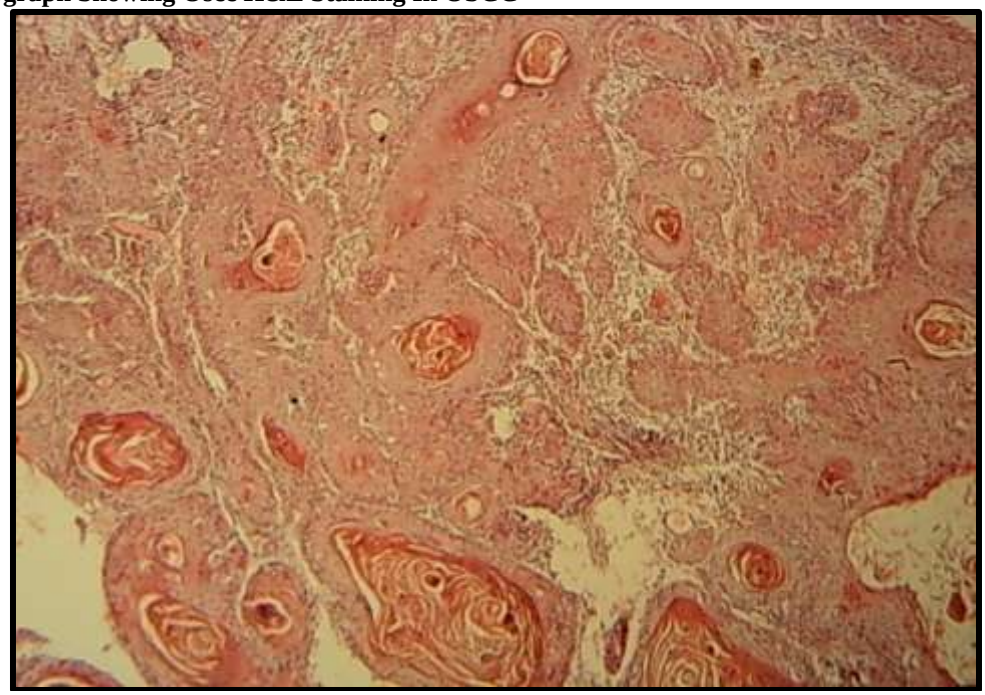
1. To access the qualitative and quantitative expression of P16 in Human Papilloma Virus positive cases.
2. To access the qualitative and quantitative expression of P16 in Human Papilloma Virus negative cases.
3. To compare the expression of P16 in Human Papilloma Virus positive and negative cases.

Materials And Method:-

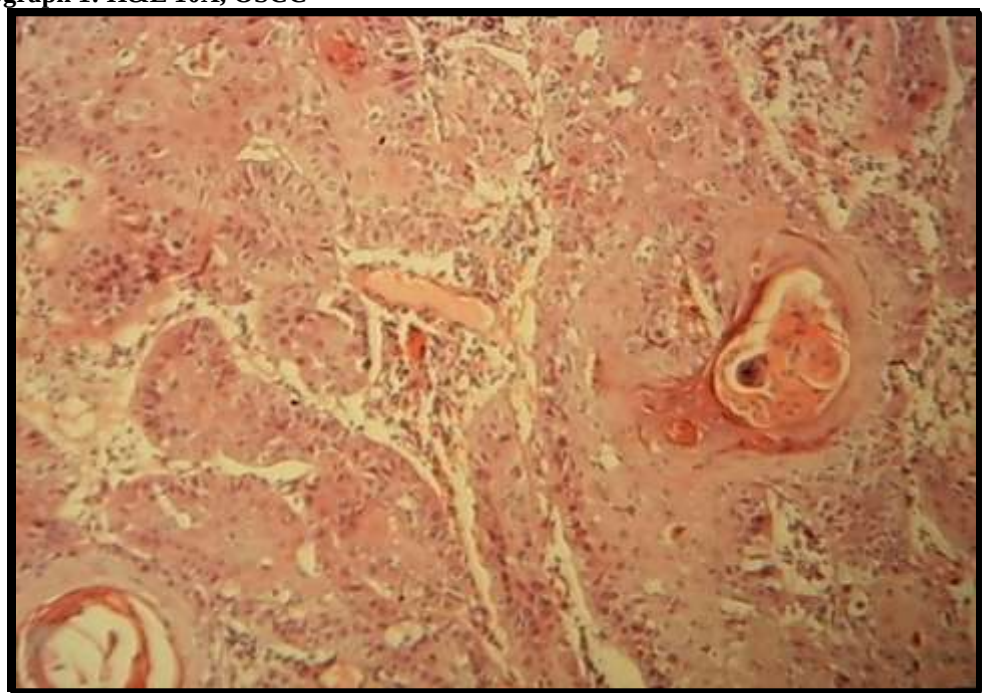
This retrospective study has been conducted to evaluate the expression of p16 in HPV positive and HPV negative OSCC using Immunohistochemistry (IHC) technique in formalin fixed paraffin - embedded (FFPE) tissues. The tissue specimens for the study comprised of a total of 60 FFPE blocks retrieved from the archive of Department of Pathology, NECHRI, Guwahati, Assam. The archive material retrieved comprised of total 60 cases of HPV positive and HPV negative oral squamous cell carcinoma (30 of each type) on which p16 IHC study was conducted. And also 10 cases of colon carcinoma were taken as positive control. Institutional ethetical clearance was obtained. Histopathologically diagnosed cases of OSCC with known Human Papilloma Virus status were included in the study whereas the samples of the patients who have undergone chemotherapy or radiotherapy were excluded. Anti -p16 (INK4) antibody (BioGenex) a**t 2 degree**was considered as the primary antibody. The Secondary antibody selected was biotinylated anti-immunoglobulins /super enhancer kept at room temperature, in humid chamber for 30 minutes. The paraffin sections were stained with Hematoxilin and eosin stain. The microscopic slides were dipped in methanol for drying and coated with poly-L-Lysine solution. The paraffin embedded tissue blocks were sectioned approximately 4 microns thick using semi -automated rotary microtome. Freshly prepared buffer were used every time before the immunohistochemical procedure to maintain the desired pH value. These are useful in immunohistochemistry for removing excess or umwanted agents or complexes formed during each step. Most common wash buffer commercially available are PBS (Phosphate Buffered saline). Tris EDTA buffer of pH 9 is used in heat induced antigen retrieval method in a microwave vessel. Following this the tissue sections were deparaffinized by heating on the slide warming table at 60degree C for 15 minutes and placed into the specific containers for the required time. Now, prior to application of primary antibody endogenous peroxide block should be used if it is necessary to block endogenous peroxide activity in the tissue being stained, when Horseradish Peroxide (HRP) is used as the labelling enzymes. Whereas power block is used for reducing non specific agents in immunoassay. It is a universal block, suitable for use in immunohistochemistry, immunogold techniques, and ELISA methods. It also works well as an antibody diluent and washing medium. In the next step the tissue sections

were incubated with few drops of primary antibody in a humidified chamber at 4 degree centigrade overnight. From this step onwards the slides were never allowed to dry, during intervals they were immersed in Tris Buffer Solution (TBS) in trough. Secondary antibody is isolated from the root of the horseradish plant. Horse Radish plant has an iron - containing haeme group (haematin) as its active site and solution is brown coloured.

Photomicrograph Showing Oscc H&E Staining In OSCC

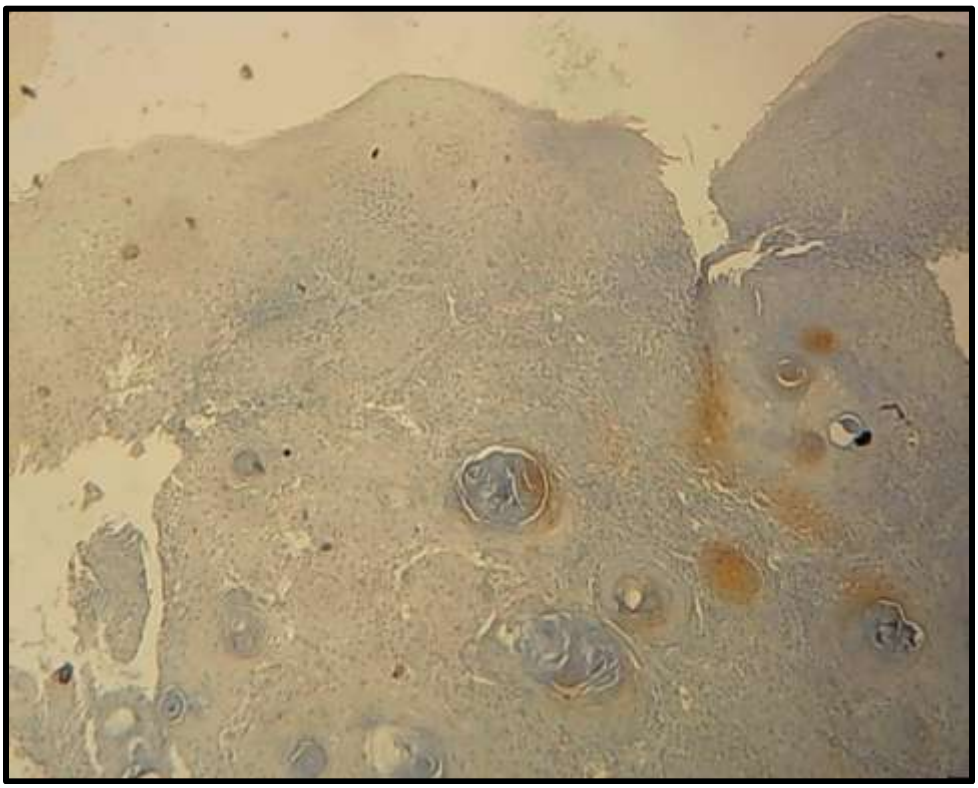


Photomicrograph 1: H&E 10X, OSCC

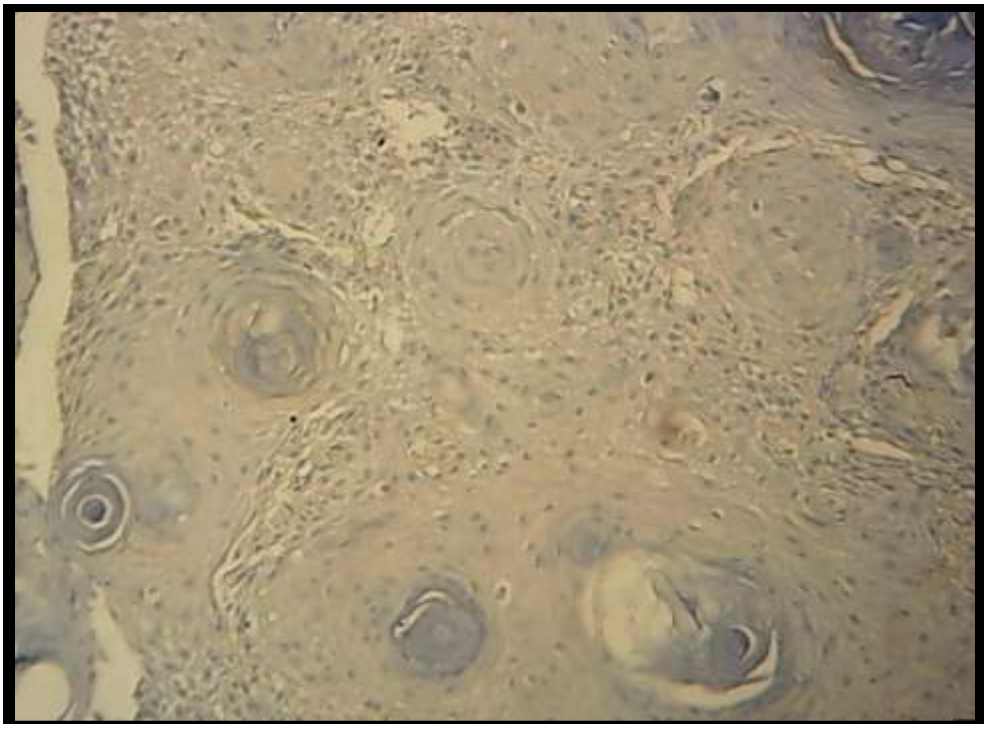


PHOTOMICROGRAPH 2: H&E, 40X OSCC

PHOTOMICROGRAPH SHOWING IHC P16 IN HPV NEGATIVE OSCC CASES

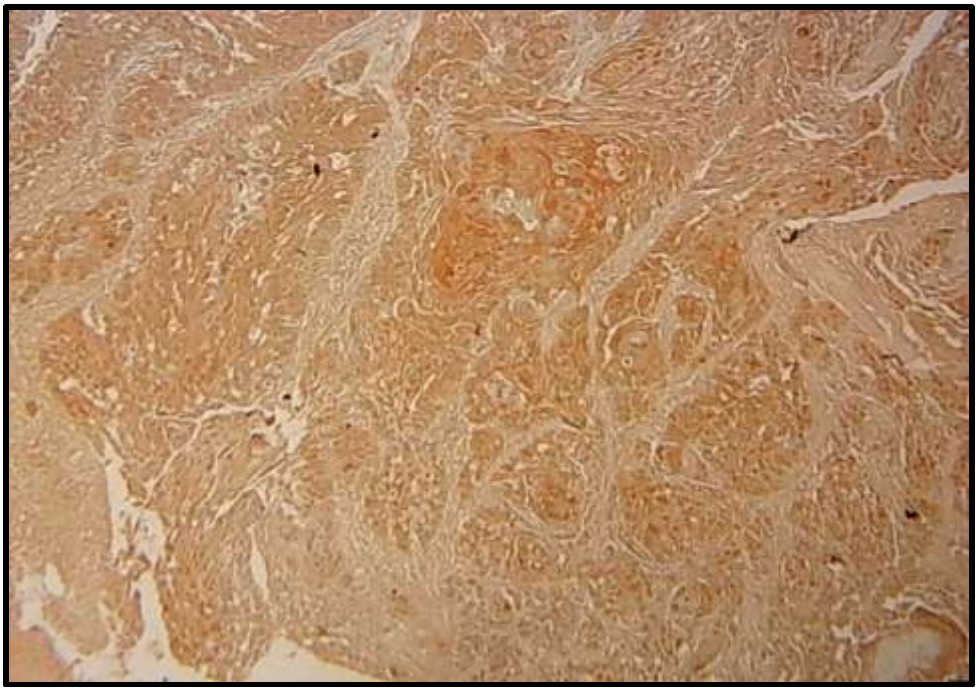


PHOTOMICROGRAPH 3: IHC 10X, HPV NEGATIVE OSCC

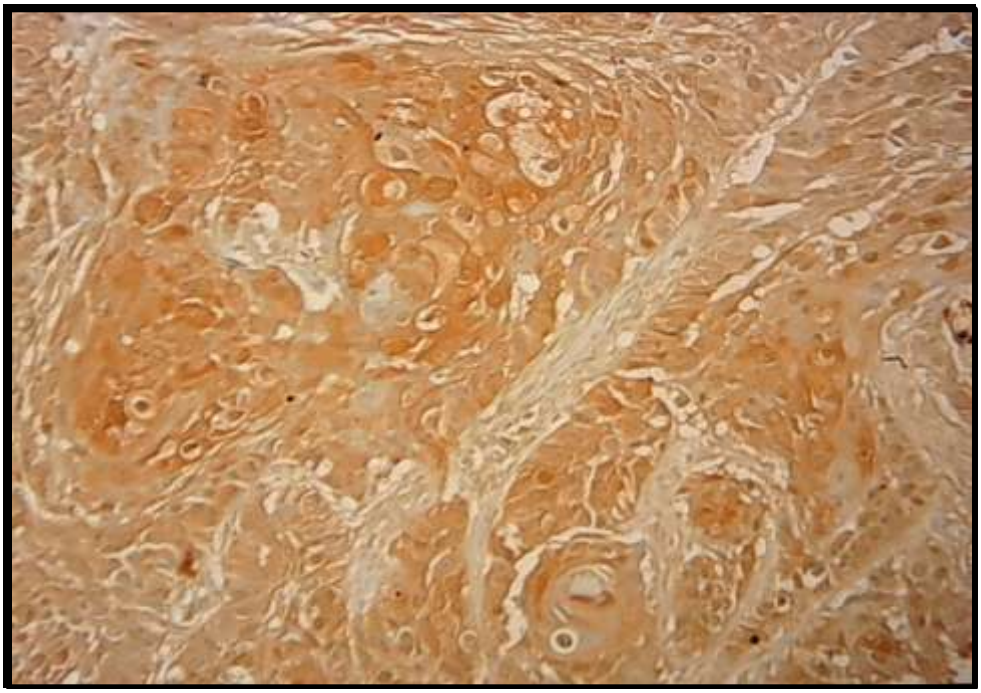


PHOTOMICROGRAPH 4: IHC, 40X NEGATIVE OSCC

PHOTOMICROGRAPH SHOWING P16 IHC IN HPV POSITIVE OSCC CASES



PHOTOMICROGRAPH 5: IHC 10X, HPV POSITIVE OSCC



PHOTOMICROGRAPH 6: IHC, 40X POSITIVE OSCC

Gender Distribution Of Study Subjects

	Female	Male
HPV -	7	23
	23.3%	76.7%
HPV +	8	22
	26.7%	73.3%

Table 1:- Age and Gender distribution in HPV +ve and HPV –ve cases in OSCC.

Site Distribution Of Study Subjects

	Alveolus	Buccal Mucosa	Labial Mucosa	Mandible	Oropharynx	Retromolar Area	Tongue	Tonsil
HPV -	8	2	4	1	9	0	5	1
	26.7%	6.7%	13.3%	3.3%	30.0%	.0%	16.7%	3.3%
HPV +	5	7	5	1	2	2	5	3
	16.7%	23.3%	16.3%	3.3%	6.7%	6.7%	16.7%	10.0%

Table 2:- Site distribution in HPV +ve and HPV –ve cases in OSCC.

Quantitative Analysis

HPV Status	Mean	Std. Deviation	Std. Error Mean	P value
HPV -	7.133	8.139	1.486	0.001 (Sig)
HPV +	61.00	25.603	4.674	

Table 3:- Mean value of p16 (Quantitative analysis) in HPV +ve and HPV –ve cases in OSCC.

Overall Staining Intensity

	Score 1	Score 2	Score 3	Chi Sq	P value
HPV -	24	6	0	35.691	0.001 (Sig)
	80.0%	20.0%	.0%		
HPV +	3	8	19		
	10.0%	26.7%	63.3%		

Table 4:- Intensity of staining (Qualitative analysis) in HPV +ve and HPV –ve cases in OSCC.

Semi Quantitative Analysis

	Diffuse	Focal	Sporadic	Negative	Chi Sq	P value
HPV -	0	2	4	24	48.121	0.001
	.0%	6.7%	13.3%	80.0%		
HPV +	19	9	2	0		
	63.3%	30.0%	6.7%	.0%		

Table 5:- Percentage of cells with staining (Semi Quantitative analysis) in HPV +ve and HPV –ve cases in OSCC.

H SCORE

	Low	High	Chi sq	P value
HPV -	30	0	25.714	0.001
	100.0%	.0%		
HPV +	12	18		
	40.0%	60.0%		

Table 6:- H-score in HPV +ve and HPV –ve cases in OSCC.

HS Core Value

HPV Status	Mean	Std. Deviation	Std. Error Mean	P value
HPV -	9.50	14.03	2.56	0.001 (Sig)
HPV +	168.53	95.51	17.43	

Table 7:- H-score core value in HPV +ve and HPV –ve cases in OSCC.

Results:-

Based on the gender distribution among the HPV- subjects 76.7 % were the males and 23.3 % were the females. Among the HPV + subjects 73.3% were the males and 26.7% were the females (Table1)

Table 2 reports based on the site of distribution among the HPV- oropharynx (30.0%) was the most predominant site followed by alveolus (26.7%), tongue (16.7%), labial mucosa (13.3%) and Buccal mucosa (6.7%). Among the HPV+ Buccal mucosa (23.3%) was the most predominant site followed by alveolus (16.7%), tongue (16.7%), labial mucosa (13.3%) and tonsil (10.0%) .

Table 3 shows the quantitative analysis among the HPV – subjects the mean number of P16 cells was 7.133 with SD of 8.139 whereas among the HPV+ subjects the mean number of P16 cells were 61.00 with sd of 25.603. The difference between HPV+ nnd HPV- was statistically significant.

Table 4 shows the overall staining intensity.Based on the staining intensity among the HPV – the staining intensity score was 1 in 80 percent of the subjects whereas in the HPV+ 63.3% were having Score 3 and 26.7% were having Score 2. The difference between HPV+ and HPV- was statistically significant when analyzed using chi square test.

Table 5 shows the results based on the percentage of p16 based on the semi quantitative analysis .Among the HPV – the staining was negative in 80 percent of the subjects whereas in the HPV+ 63.3% were having diffuse staining and 30.0% were having focal staining. The difference between HPV+ and HPV- was statistically significant when analyzed using chi square test

Table 6 shows the results based on H score which shows that among the HPV – the 100 percent of the subjects were having low H Score whereas in the HPV+ 60.0% were having high H score and 40.0% were having low H score The difference between HPV+ and HPV- was statistically significant when analyzed using chi square test.

Table 7 reports that among the HPV – subjects the mean H score was 9.50 with SD of 14.03 whereas among the HPV+ subjects the mean H score was 168.53 with sd of 95.51. The difference between HPV+ nnd HPV- was statistically significant when analyzed using the independent t test .

Discussion:-

The choice of a suitable method for detection of HPV DNA has become increasingly complex (De Villiers1997).Whereas PCR has been considered the most appropriate method for viral detection ,IHC is widely used in routine diagnosis or scientific investigations .Although considered a less sensitive method, IHC has proven to be a very important molecular tool in diagnosis and research and has significantly advanced the study of gene structure and expression at the level of individual cells. Functional loss of p16 has been reported for many human cancers, whereas in HPV-associated cervical carcinomas p16 overexpression has been observed. This overexpression is frequently related to high-risk HPV infections, and p16 immunohistochemical expression has been used as a substitute marker for HPV presence.

In this study it has been demonstrated that p16 immunohistochemical staining of neoplastic cells may be used as a surrogate marker for HPV status of OSCC. Using previously described grading criteria to evaluate p16 immunoreactivity revealed a highly significant correlation of p16 and HPV status. The purpose of the study was to evaluate the expression of p16 in the form of percentage of cells and the intensity of staining in both HPV +ve and –ve OSCC cases. The H scoring criteria was applied for both the cases and the value was obtained by multiplying the percentage of positive cells and their staining intensities for both type of cases.Out of 60 samples, 30 cases were of HPV positive and 30 cases were of HPV negative oral squamous cell carcinoma patient. With respect to the gender and age distribution, among all the 60 cases included, 43 cases were of male patients and 17 were of female patients and the age distribution ranged from 40 to 70 years. The gender distribution showed that majority of the study groups are males ie, 73.3% were HPV +ve and 76.7% were HPV –ve cases, when compared to females ie, 26.7% were HPV+ve cases and 23.3% were HPV –ve cases. **(Chen C.J et al 2012).**Based on the site of distribution among the HPV- oropharynx (30.0%) was the most predominant site followed by alveolus (26.7%), tongue (16.7%), labial mucosa (13.3%) and Buccal mucosa (6.7%). Among the HPV+ Buccal mucosa (23.3%) was the most predominant site followed by alveolus (16.7%), tongue (16.7%), labial mucosa (13.3%) and tonsil (10.0%). Hence it suggested that HPV is primarily associated with oropharyngeal cancers. These findings were similar to the study done by **Fakhry C et al, 2008,** although the generalized proportions of oropharynx cancer attributable to HPV infection is unclear.When quantitative analysis was done, among the HPV – subjects the mean number of P16 cells was 7.133 with SD of 8.139 whereas among the HPV+ subjects the mean number of P16 cells were 61.00 with sd of 25.603. The difference between HPV+ nnd HPV- was statistically significant. Based on the semi quantitative analysis among the HPV – the staining was negative in 80 % of the subjects whereas in the HPV+ 63.3% were having diffuse staining and 30.0% were having focal staining. The difference between HPV+ and HPV- was statistically significant when analyzed using chi square test The findings were correlated to study done by **Fregonesi PAG et al, 2003.**Intensity of staining was evaluated for both nucleus and cytoplasm for HPV +ve and HPV -ve cases. Based on the staining intensity among the HPV – the staining intensity score was 1 in 80 percent of the subjects whereas in the HPV+ 63.3% were having Score 3 and 26.7% were having Score 2. The difference between HPV+ and HPV- was

statistically significant when analyzed using chi square test. Thus high intensity staining was recorded in HPV +ve cases whereas low intensity staining was found in HPV –ve cases. The probable reason for this might be because of the overexpression of p16 in HPV +ve cases when compared to HPV –ve cases. Our findings indicated the presence and the possible role of HPV in carcinogenesis which is in accordance to the study by **Balaram et al(1995)**who reported 74% HPV presence in OSCC using p16 immunostaining. Another study by **Patil et al(2006)**using p16 immunostaining for detection of HPV in OSCC found 87% positive cases. For calculation of H-score both Nuclear and cytoplasmic staining intensity was evaluated. Intensity of staining was scored into no staining (0), weak (1+), intermediate (2+), strong (3+) while percentage of positively stained cells were scored from 0 to 100. Intensity and percentage scores were multiplied to calculate a total H- score. An H- score < 200 was considered as low p16 expression while an H-score > 200 was considered as positive staining for p16 expression **(Hashmi A.A et al,2019).** In the present study, based on the H Score among the HPV –ve, 100 % of the subjects were having low H Score whereas in the HPV+ 60.0% were having high H score and 40.0% were having low H score The difference between HPV+ and HPV- was statistically significant when analyzed using chi square test. Among the HPV – subjects the mean H score was 9.50 with SD of 14.03 whereas among the HPV+ subjects the mean H score was 168.53 with sd of 95.51. The difference between HPV+ nnd HPV- was statistically significant when analyzed using theindependent t test. A distinct staging algorithm for HPV associated oropharyngeal cancer (OPC) has been included in the 8th edition of American Joint Committee om Cancer (AJCC), wherein P16 overexpression is a criteria to designate HR-HPV associated OPC **(Sudhakaran A et al, 2019).** As per the criteria, p16 overexpression is determined by intense staining and > 75% cut- off. Various studies have also shown p16 to have a sensitivity of 74% to 100% and specificity of 46% to 100% when compared with other methods **(Larsen CG et al, 2014).** Authors have also found high specificity and sensitivity with p16 immunostaining in comparison to other methods like ISH **(Schlecht NF et al, 2011) .**

Materials and Method:-

Forty caries-free healthy institutionalized children in the age group 6-12 years were selected based upon dental examination and relevant case history. Children in this age group have mixed dentition which is a highly dynamic stage as the primary teeth are exfoliating and the permanent teeth are erupting, so this transition period usually have increased susceptibility to dental caries. Consent for participation in the study was obtained. Children having dmft/DMFT scores as 0(zero),according to WHO diagnostic criteria for dental caries, and residing in the institution's hostel thus having similar dietary and oral hygiene habits, were included in the study for proper monitoring and standardization purpose. Unstimulated saliva samples were collected at baseline from the participants before the clinical trial to establish Streptococcus mutans level as well as salivary pH. The children were explained the importance of good oral health and demonstrated with correct method of tooth brushing by a trained professional. All of them were provided with commercially available, conventional and similar tooth pastes and tooth brushes at the beginning of the study. The participants were randomly divided into two groups- Group A and Group B of 20 children each. Group A or Experimental group children were advised to chew oral Immunoglobulin Y (Ig Y) containing 'No decay' table twice daily, as per recommended dose ie, one 20 mg chewtab (orange in color) in the morning after breakfast and one 40 mg chew tab (white in color) in the night after dinner, for a intervention period of 15 days. As per the manufacturer's guidelines, these children were also advised to continue their regular tooth brushing, twice daily, along with using the tablets, but following correct tooth brushing technique as demonstrated. They were asked not to swallow but chew the tablets, brush their teeth properly before chewing and neither eat nor drink anything for at least half an hour after chewing. Group B or Control group children, were advised to do only regular tooth brushing, twice daily for 15 days, following correct tooth brushing technique. Group B children were not given the Ig Y containing chew tabs. Compliance and effectively of tooth brushing was ensured with the study being performed under supervision. Unstimulated saliva samples were collected again from the participants at the end of intervention period. Saliva samples from both the Groups A and B were again divided into subgroups of 10 each, ie, A1(n=10), A2(n=10) and B1(n=10), B2(n=10). Samples from A1 and B1 were tested for salivary Streptococcus mutans count whereas samples from A2 and B2 were tested for salivary pH changes[Figure 2].The saliva samples were coded during sample collection and microbiological analysis. After being transported in a thermocol ice box to lab for investigation, the saliva samples were immediately subjected for microbiological analysis to assess the S.mutans level. The saliva samples were diluted in phosphate buffered saline (pH 7.0) to the serial dilutions of 1/103, agitated for30 seconds on a vortex cyclomixer. One milliliter of saliva sample was inoculated into mites salivarius bacitracin agar(MSB). The agar plates were incubated at 37°C for 48 hours under anaerobic conditions. S.mutans colonies were identified as spherical, raised and dark blue in colour [Figure 3]. The number of S.mutans colonies per milliliter of saliva on each plate was enumerated using the colony counter [Figure 4]. Following this the mean colony forming units (CFU/ml) was determined and semi quantification

of the number of colonies was done by multiplying the actual colony count with its dilution factor. Salivary pH was measured with the help of a handheld digital pH meter for both the groups. The mean percentage change (between baseline and post intervention) in salivary S.mutans count as well as salivary pH respectively, were evaluated for both the groups and compared with each other using paired and unpaired student 't' test. The data obtained was tabulated and statistically analyzed. Significance level was fixed at $P < 0.05$.

Conclusion:-

The present study revealed that p16 can be used as a surrogate marker for HPV in OSCC. Furthermore p16 IHC is a rapid , easy, inexpensive and reliable test and thus as a research approach p16 can be used as a screening test for HPV in these subsites with a lower prevalence of HPV to rule out the presence of HPV .In our results it was confirmed that p16 is highly correlated with HPV status in OSCC .In future studies with larger samples p16 could be a discriminator that is easily applicable in a routine pathology and become the basis of treatment decisions based on histological features rather than on staging alone.

References:-

1. Johnson DE, Burtness B, Leemans CR, Lui VWY, Bauman JE, Grandis JR. Head and neck squamous cell carcinoma. Nat Rev Dis Primers. 2020;6(1)
2. Joshi P, Dutta S, Chaturvedi P, Nair S. Head and neck cancers in developing countries. Rambam Maimonides Med J. 2014;5(2)
3. Psyrri A, Gouveris P, Vermorken JB. Human papillomavirus-related head and neck tumors: clinical and research implication. Curr Opin Oncol. 2009;21(3):201-205.
4. Gillison ML, Koch WM, Capone RB, et al. Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. J Natl Cancer Inst. Wang W, Han S, Yao Z, Li X, Huang P, Zhang M, Chen Y, He J. A study of epidemiologic and recurrence factors of oral cancer. Journal of oral and maxillofacial surgery. 2012 Sep 1;70(9):2205-10.
5. De Matos LL, Trufelli DC, De Matos MG, da Silva Pinhal MA. Immunohistochemistry as an important tool in biomarkers detection and clinical practice. Biomarker insights. 2010 Jan;5:BMI-S2185.
6. Kim SM, Kwon IJ, Myoung H, Lee JH, Lee SK. Identification of human papillomavirus (HPV) subtype in oral cancer patients through microarray technology. European Archives of Oto-Rhino-Laryngology. 2018 Feb;275(2):535-8
7. Sudhakar A. History of cancer, ancient and modern treatment methods. Journal of cancer science & therapy. 2009 Dec 1;1(2):1.
8. Hajdu, S. I. A note from history: Landmarks in history of cancer, part 1. Cancer. 2011;117(5):1-12
9. Hajdu SI. A note from history: landmarks in history of cancer, part 2. Cancer. 2011;117(12):2811-2820
10. Gillison ML, Koch WM, Capone RB, et al. Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. J Natl Cancer Inst. 2000;92(9):709-720.
11. Chang F, Syrjänen S, Kellokoski J, Syrjänen K. Human papillomavirus (HPV) infections and their associations with oral disease. J Oral Pathol Med. 1991;20(7):305-317.
12. Gillison ML, D'Souza G, Westra WH, Sugar E, Xiao W, Begum S et al. Distinct risk factor profiles for human papillomavirus type 16-positive and human papillomavirus type 16-negative head and neck cancers. Journal of the National Cancer Institute. 2008 Mar;100(6):407-420. 27
13. Ram H, Sarkar J, Kumar H, Konwar R, Bhatt ML, Mohammad S. Oral cancer: risk factors and molecular pathogenesis. J Maxillofac Oral Surg. 2011;10(2):132-137.
14. Chi AC, Day TA, Neville BW. Oral cavity and oropharyngeal squamous cell carcinoma--an update. CA Cancer J Clin. 2015;65(5):401-421.
15. Harris SL, Thorne LB, Seaman WT, Hayes DN, Couch ME, Kimple RJ. Association of p16(INK4a) overexpression with improved outcomes in young patients with squamous cell cancers of the oral tongue. Head Neck. 2019;33(11)
16. Fakhry C. Special Issue about Head and Neck Cancers: HPV Positive Cancers. Int J Mol Sci. 2020;21(9):3388.
17. Shapiro M, Salama A. Margin Analysis: Squamous Cell Carcinoma of the Oral Cavity. Oral Maxillofac Surg Clin North Am. 2017;29(3):259-267.
18. Bugshan A, Farooq I. Oral squamous cell carcinoma: metastasis, potentially associated malignant disorders, etiology and recent advancements in diagnosis. F1000Res. 2020;9:229.

19. Daniel E. Johnson1, Barbara Burtness. Head and neck squamous cell carcinoma. Nat Rev Dis Primers. 2021 ; 6(1): 92.
20. Götz C, Bischof C, Wolff KD, Kolk A. Detection of HPV infection in head and neck cancers: Promise and pitfalls in the last ten years-a meta-analysis. Molecular and Clinical Oncology. 2019 Jan 1;10(1):17-28.
21. Plath M, Broglie MA, Förbs D, Stoeckli SJ, Jochum W. Prognostic significance of cell cycle-associated proteins p16, pRB, cyclin D1 and p53 in resected oropharyngeal carcinoma. Journal of Otolaryngology-Head & Neck Surgery. 2018 Dec;47(1)
22. Gabrielli Fregonesi PA, Teresa DB, Duarte RA, Neto CB, de Oliveira MR, Soares CP. p16INK4A immunohistochemical overexpression in premalignant and malignant oral lesions infected with human papillomavirus. Journal of Histochemistry & Cytochemistry. 2003 Oct;51(10):1291-7.
23. J Kales ,Sritippho T, Pongsiriwet S, Lertprasertsuke N, Buddhachat K, Sastraruji T, Iamaroon A. p16-a possible surrogate marker for high-risk human papillomaviruses in oral cancer. Asian Pac J Cancer Prev. 2016 Jan 1;17(8):4049-57.
24. Hashmi AA, Hussain ZF, Irfan M, Hashmi SK, Asif H, Faridi N. Prognostic significance of p16 immunohistochemical expression in urothelial carcinoma. Surgical and Experimental Pathology. 2019 Dec;2(1):1-8.
25. C. Babu, Danaei G, Riley LM, Paciorek CJ, Stevens GA, Gregg EW, Bennett JE, Solomon B, Singleton RK, Sophiea MK. Worldwide trends in hypertension prevalence and progress in treatment and control from 1990 to 2019: a pooled analysis of 1201 population-representative studies with 104 million participants. The Lancet. 2021 Sep 11;398(10304):957-80.
26. Bilde A, Von Buchwald C, Dabelsteen E, Therkildsen MH, Dabelsteen S. Molecular markers in the surgical margin of oral carcinomas. Journal of oral pathology & medicine. 2009 Jan;38(1):72-8.
27. Sharma SJ, Wagner S, Reder HS, Kroll T, Wuerdemann N, Klussmann JP, Wittekindt C. The 8th edition AJCC/UICC TNM staging for p16-positive oropharyngeal carcinoma: is there space for improvement?. European Archives of Oto-Rhino-Laryngology. 2018 Dec;275(12):3087-91
28. Patil S, Rao RS, Amrutha N, Sanketh DS. Analysis of human papilloma virus in oral squamous cell carcinoma using p16: An immunohistochemical study. Journal of international society of preventive & community dentistry. 2014 Jan;4(1)
29. El-Mofty SK, Patil S. Human papillomavirus (HPV)-related oropharyngeal nonkeratinizing squamous cell carcinoma: characterization of a distinct phenotype. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology. 2006 Mar 1;101(3):339-45.
30. Balaram P ,Herrero R, Castellsagué X, Pawlita M, Lissowska J, Kee F, Rajkumar T, Sridhar H, Rose B, Pintos J, Fernández L. Human papillomavirus and oral cancer: the International Agency for Research on Cancer multicenter study. Journal of the National Cancer Institute. 2003 Dec 3;95(23):1772-83.