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Original Article

Immunohistochemical (IHC) Expression of p16 in Various Grades of Oral Squamous Cell Carcinoma (OSCC) with Snuff Use in Tertiary Care Hospitals of Peshawar

Karishma Ali[1*], Sadaf Alam[1], Natasha Kamran[2], Sabeen Nasir[1], Razia Aamir[2] and Sarah Yousuf[1]

[1]Department of Pathology, Peshawar Medical College, Affiliated with Riphah International University, Islamabad, Pakistan
[2]Department of Pathology, Peshawar Dental College, Affiliated with Riphah International University, Islamabad, Pakistan

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***Corresponding Author:**
Karishma Ali
Department of Pathology, Peshawar Medical College, Affiliated with Riphah International University, Islamabad, Pakistan
karishmasiki@gmail.com

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ABSTRACT

Oral squamous cell carcinoma (OSCC) is a significant health concern, with various factors influencing its development and progression. Understanding these factors, including p16 expression and clinicopathologic features, is crucial for improved diagnosis and treatment. **Objective:** To compare various grades of OSCC based on immunohistochemical expression of p16 and clinicopathologic parameters. **Methods:** The comparative cross-sectional study was conducted at the Department of Pathology, Peshawar Medical College (PMC), and Peshawar Dental College (PDC) from August 2020 to August 2021. It included 53 cases of OSCC with documented snuff use history. Tumor sections were stained with Hematoxylin and Eosin and underwent immunohistochemical staining for p16 expression. Sample size calculation utilized G Power software. Statistical analysis was performed using SPSS version 20.0, employing the Chi-Square test to assess categorical variables. **Results:** Among the 53 OSCC cases, the majority were male (66.0%) with ages ranging from 26 to 85 years, the most common age group being 51-70 years. The tongue was the primary site for OSCC development. Well-differentiated cases were predominant (64.2%), followed by moderate (20.8%) and poor grade cases (15.1%). However, no significant association was found between p16 expression and OSCC grades. Notably, p16 expression tended to be higher in snuff users and well-differentiated OSCC cases, although not statistically significant. **Conclusions:** Well-differentiated OSCC cases exhibited the highest expression of p16, followed by moderate and poorly differentiated cases. However, no significant correlation was observed between p16 expression and OSCC in snuff users.

INTRODUCTION

According to GLOBOCAN estimates from 2020, Head and Neck Squamous Cell Carcinoma (HNSCC) ranks as the seventh most common cancer globally. These estimates also indicate that HNSCC leads to approximately 450,000 deaths annually, accounting for roughly 4.6% of all cancer-related deaths worldwide [1]. Additionally, there are around 890,000 new cases of HNSCC diagnosed each year, constituting about 4.5% of all cancer diagnoses worldwide. Oral cancer represents a substantial burden of morbidity and mortality, particularly prevalent in Central and Eastern Europe, Melanesia, and South Asia [2]. As of 2018, the incidence, prevalence, and distribution of oral cancer vary significantly across regions worldwide. Notably, the ten most populous countries globally China, India, the United States, Indonesia, Brazil, Pakistan, Bangladesh, Russia, Japan, and Mexico are dispersed across Asia, North America, South America, and Europe, collectively comprising 56.3% of the total world population. Among these nations, India, Pakistan, and Bangladesh are particularly affected by oral cancer, reflecting significant health challenges within these regions [3]. Research reported in Karachi revealed that OSCC accounted for 8.8% of all cancers in that region. Despite advancements in cancer diagnosis and treatment over the last three decades, the overall 5-year survival rate for OSCC remains notably low, consistently falling below 50% [4]. Globally,

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Oral squamous cell carcinoma (OSSC) is more common in men's than in women that is 2:1[5]. OSCC was believed to be more prevalent in individuals aged 60 to 80 years. OSCC can manifest in various areas of the "oral cavity" including the, tongue, mucosa of the buccal area, palate, floor of the mouth, lip, and even the gingiva. Globally, the tongue is the commonest site for OSCC, while in South Asia, particularly, the mucosa of buccal area is more frequently affected [6]. Nicotine use, chewing of pan, and prolonged exposure to sun are frequently cited as common etiological factors for OSCC. In addition to these, both smokeless tobacco (snuff) and Human Papillomavirus (HPV) have been identified as factors that elevate the risk of developing OSCC [7,8]. The direct contact of snuff with the oral mucosa induces keratinization of the oral epithelium. This keratinized epithelium sets the stage for premalignant lesions through various signaling pathways, ultimately progressing to malignancy [9]. Snuff exerts a dual impact by suppressing and inflaming immune cells, thereby fostering autoimmunity. These effects collectively contribute to the development of oral malignancies [10]. The expression of HPV genes can be influenced by certain environmental chemical compounds. A noteworthy correlation exists between HPV and tobacco-related carcinogens, particularly in the context of oral cancer [11]. Two specific carcinogens present in snuff, namely nitrosamines and nitroguanosine, induce a tumor phenotype in primary oral keratinocytes that have been immortalized with HPV. Benzo[α] pyrene (BaP), another carcinogen found in snuff, leads to a tenfold increase in HPV titers. Additionally, snuff exhibits an immunosuppressive effect against HPV [12]. In the pathogenesis of OSCC, high-risk HPV types such as HPV16 and 18 play a significant role, as highlighted by Khokhar *et al* [13]. It's noteworthy that "patients with HPV-associated OSCC" tend to have a prognosis that is 90% more favorable compared to those without HPV-associated OSCC. The development of HPV-associated oral squamous cell carcinoma (OSCC) encompasses a diverse array of genetic mutations, deletions, and translocations. Notably, prominent among these alterations are p16, p53, cyclin D1, p63, PTEN, Rb, and the Epidermal Growth Factor Receptor [14]. While the primary role of p16 is to hinder the tumor process, gene mutations can contribute to the onset of OSCC. As such, p16 holds promise as a potential biomarker for predicting high-risk HPV-associated OSCC [15]. In normal cells, p16 is expressed at low levels, making it nearly undetectable through immunohistochemistry (IHC) [16]. However, due to the transformative activity of the E7 genes, p16 exhibits high expression in tumor cells infected by HPV, facilitating its easy detection. Consequently, there exists a close association between p16 expression and HPV positivity [17].

The aim of study was to find out p16 expression in OSCC in snuff users, since the snuff affects the immune system adversely, which makes patients an easy target for viral infection.

METHODS

This cross-sectional study with a descriptive design was conducted at the Department of Pathology, Peshawar Medical and Dental College in Peshawar. The study spanned a period of one year from August 2020 to August 2021. The sample size has been calculated by using the G power software with (3.5%) prevalence of oral squamous cell carcinoma in KP. Employing a one-tailed test with an effect size of 0.5, an α value of 0.05 (indicating the margin of type 1 error), and a power of 80% (with a minimum acceptable probability for type 2 error set at 20%). Statistical analysis was conducted using the Statistical Package for Social Sciences (SPSS) version 20.0. Categorical variables were assessed using the Chi-Square test. "The sample size was determined using G Power software version (3.1.9.7) with an effect size of 0.5, alpha set at 0.05, power at 80%, and a degree of freedom of 2%". Sampling for this study was performed using a nonprobability convenient sampling technique. The ethical guidelines were followed and approval was received from the "Institutional Review Board (IRB)" in Peshawar, with the assigned approval number being "Prime/IRB/2020/235". The study included 53 previously diagnosed cases of oral squamous cell carcinoma (OSCC). Exclusions comprised cases with poor formalin fixation, which may lead to antigen retrieval issues, and blocks from patients undergoing chemotherapy. Cases with accessible historical records were collected from the "Department of Pathology" at "Peshawar Medical College (PMC)" and the Department of Surgery at "Peshawar Dental College and Hospital (PDC)". Immunohistochemical staining was performed at the "Department of Pathology, Peshawar Medical College, Riphah International University" laboratory, while all other laboratory procedures were conducted at the Department of Histopathology, Peshawar Medical College. Tissue blocks embedded in paraffin and treated with formalin from previously diagnosed cases of oral OSCC. Sections were cut from these blocks for both Hematoxylin and Eosin (H&E) staining and immunohistochemistry (IHC) procedures. Thin sections, measuring 4 to 5 µm of the OSCC with paraffin embedded tissue were subjected to H&E staining following standard protocols. The diagnosis of oral cancer and immunohistochemistry staining with (p16) antibody involved microscopic examination of Hematoxylin and Eosin (H&E) slides. The interpretation of p16 expression was conducted manually, involving several steps. First, the World Health Organization (WHO) guideline for histopathological grading of OSCC was utilized, categorizing OSCC into three grades: well-differentiated (Grade 1), moderately differentiated (Grade 2), and poorly

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differentiated (Grade 3). Next, scoring of p16 intensity was based on the observed staining, with a scale of 1 to 3 indicating weak, moderate, and strong expression, respectively. Additionally, the percentage of stained cells was assessed, score 0: No staining, score 1: 0-10% of cells stained, score 2: 11-50% of cells stained, score 3: 51-80% of cells stained, score 4: 81-100% of cells stained. The assessment considered the intensity and percentage of brown staining of cells (cytoplasmic and nuclear staining) under microscopic examination, with stronger intensity indicating higher p16 expression and weaker intensity suggesting lower expression. At last, the p16 end score, ranging from 0 to 12, was derived by multiplying the intensity and percentage of stained cells (intensity of stained cell x percentage of stained cells). A score of 4 or higher indicated positive p16 expression.

The method of indirect immunohistochemistry was used. Tissues fixed in formalin and embedded in paraffin were subjected to deparaffinization. The retrieval of antigens was achieved by immersing the sample in a citrate buffer solution, followed by heating in an oven at 95-100 degree Celsius for a duration of 20 minutes. The slides were permitted to cool at room temperature for a period of 15 to 20 minutes. Slides were rinsed using a phosphate-buffered saline (PBS) and distilled water. The sections of the slides were treated with a peroxidase blocking solution (PBS) and incubated at room temperature for a duration of 10 minutes. After rinsing the slide in Phosphate Buffer Solution for 6 minutes, the chromogen substrate was applied to reveal the color of the antibody. Subsequently, the slides were incubated in a peroxidase substrate solution. Following a color development time of less than 5 minutes, the slides were cleaned. Next, the slides were submerged in Hematoxylin counterstaining solution for a period of 1-2 minutes. and further cleaned for 15 minutes under running tap water. Tissue slides were dehydrated using alcohol for 5 minutes. The final steps involved cleaning the slides with 3 changes of xylene, applying a cover slip with a mount solution and storing the slides at room temperature.

In this comparative cross-sectional study, data analysis was conducted using SPSS software (version 25.0). For categorical data, including parameters such as p16 expression and gender, frequency and percentages were determined. Chi-square tests were utilized to analyze categorical data, with a significance level set at a P-value less than or equal 0.05. This approach allowed for the comparison of variables among the three categories of OSCC.

RESULTS

The demographic and disease history of patients with OSCC were of total 53 cases of OSCC, majority patients were males (n=35, 66.0%), and the overall age range spanned from 26 to 85 years, with the most common age range being 61-70. The predominant site of OSCC development was the tongue, followed by the buccal mucosa. The majority of OSCC cases (n = 34, 64.2%) had well-differentiated histopathological grades, followed by cases that were moderately differentiated (n = 11, 20.8%) and poorly differentiated (n = 8, 15.1%), a majority of individuals with a history of snuff use exhibited p16 positivity as outlined in (table 1).

Table 1: Basic Demographics and Disease History of Patients with OSCC

Variable	Male				Female				
OSCC in Snuff Users	35 (66.0%)				18 (34.0%)				
	Age Range								
	21-30	**31-40**	**41-50**		**51-60**	**61-70**	**71-80**	**81-90**	
	2 (3.8%)	2 (3.8%)	9 (17.0%)		14 (26.4%)	16 (30.2%)	7 (13.2%)	3 (5.7%)	
	Location of lesion								
	Tongue	**Buccal Area**	**Cheek**	**Oropharynx**	**Bone**	**Maxilla**	**Lip**	**Oral Cavity**	**Mandible**
	16 (30.2%)	13 (24.5%)	2 (3.8%)	9 (17.0%)	3 (5.7%)	1 (1.9%)	3 (5.7%)	3 (5.7%)	3 (5.7%)
	Well Differentiated OSCC			**Moderately Differentiated OSCC**			**Poorly Differentiated OSCC**		
	34 (64.2%)			11 (20.8%)			8 (15.1%)		
	Expression of p16 in OSCC								
	Negative				**Positive**				
	2 (3.77%)				51 (9.22%)				

In the 53 cases with a history of snuff usage, no significant associations were observed between gender, age, the site of OSCC lesions, and the different grades of OSCC with expression of p16 across. The calculated p-value exceeded 0.05, as indicated in Table 2A, Table 2B, Table 2C and Table 2D. The study included a total of 53 patients diagnosed with Oral Squamous Cell Carcinoma (OSCC), among whom a significant majority were male (66.0%). Notably, the age group most commonly affected was 61-70 years. Upon analysis of lesion sites, the tongue emerged as the predominant location, accounting for 30.2% of cases. Histological grading revealed that well-differentiated OSCC was the most prevalent,

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comprising 64.2% of cases. Moreover, all patients showed positive expression of p16 within the OSCC samples. These findings shed light on the demographic profile and disease characteristics of OSCC patients within the study, emphasizing the predominance of males, older age groups, and the consistent presence of p16 expression.

Table 2A: Relation of p16 Expression in OSCC Cases with Gender of the Patients

Gender	n (%)	p16 Expression		p-Value
		Positive	Negative	
Male	35 (66.0%)	33 (66.2%)	2 (3.7%)	0.3
Female	18 (33.9%)	18 (33.9%)	0 (0%)	
Total	53 (100%)	51 (96.2%)	2 (3.7%)	

Table 2B: Age Distribution of OSCC Cases with p16 Expression

Age	n (%)	p16 Expression		p-Value
		Positive	Negative	
21-30 Years	2 (3.7%)	2	0	0.11
31-40 Years	2 (3.7%)	2	0	
41-50 Years	9 (16.9%)	8	1	
51-60 Years	14 (26.4%)	14	0	
61-70 Years	16 (30.1%)	16	0	
71-80 Years	7 (13.2%)	7	0	
81-90 Years	3 (5.6%)	2	1	
Total	53 (100%)	51 (96.2%)	2 (3.7%)	

Table 2C: Relation of p16 Expression with Site Involved in OSCC Cases

Site of the Lesion	n (%)	p16 Expression		p-Value
		Positive	Negative	
Tongue	16 (30.1%)	16 (30.1%)	0 (0%)	0.9
Buccal Mucosa	13 (24.5%)	12 (22.6%)	1 (1.8%)	
Cheek	2 (3.7%)	2 (3.7%)	0 (0%)	
Oropharynx	9 (16.9%)	8(15%)	1 (1.8%)	
Bone	3 (5.6%)	3 (5.6%)	0 (0%)	
Maxilla	1 (1.8%)	1 (1.8%)	0 (0%)	
Mandible	3 (5.6%)	3(5.6%)	0 (0%)	
Lip	3 (5.6%)	3 (5.6%)	0 (0%)	
Oral Cavity	3 (5.6%)	3 (5.6%)	0 (0%)	
Total	53 (100%)	51 (96.2%)	2 (3.7%)	

Table 2D: Comparison of p16 Expression in OSCC Cases with Grades of OSCC

Grades	n (%)	p16 Expression		p-Value
		Positive	Negative	
Well Differentiated	34 (64.1%)	32 (60.3%)	2(3.7%)	0.5
Moderately Differentiated	11 (20.7%)	11 (20.7%)	0 (0 %)	
Poorly Differentiated	8 (15.0%)	8 (15.0%)	0 (0%)	
Total	53 (100%)	51 (96.2%)	9 (16.9%)	

Figure 1 (A) shows the H&E staining of 40x demonstrating well differentiated OSCC in snuff users While in figure 1 (B), immunohistochemistry p16 was applied. Expression of p16 stains (brown color staining) was strong positive in snuff users of OSCC. (black arrows). Majority of cells were brown stain which meant that well differentiated OSCC was positive for p16.

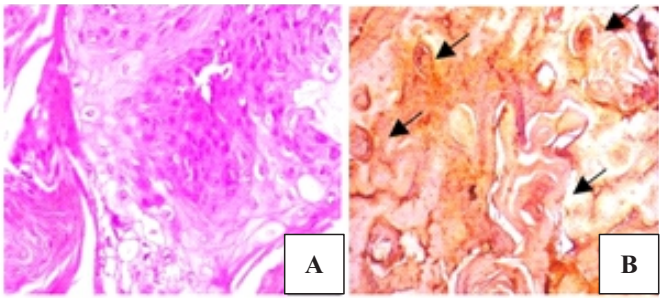

Figure 1: (A) H&E Staining of 40x, Well Differentiated OSCC; **(B)** Positive Expression of p16 Stains

Figure 2 (A) shows the H&E staining of 40x demonstrating the poorly differentiated OSCC in snuff users, while in figure 2 (B) immunohistochemistry p16 was applied. Expression of p16 stains (brown color staining, black arrows) was weak positive in snuff users of OSCC. Blue arrows show negative staining of cells in snuff users of OSCC.

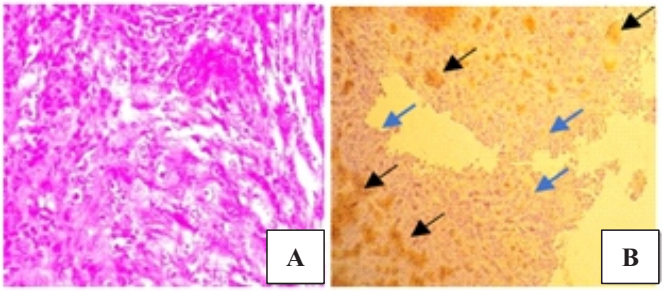

Figure 2: A) H&E Staining of 40x Poorly Differentiated OSCC; **(B)** Weak Expression of p16 Stains

DISCUSSION

Based on our results, the prevalent location for OSCC was the tongue. This aligns with the findings of a study conducted by Yosefof *et al.*, which also identified the tongue as the most frequent site for the development of OSCC [18]. The findings of investigation conducted by Ehtesham *et al.*, revealed that oral squamous cell carcinoma (OSCC) predominantly in males, involveed the floor of the mouth and the boundary of the tongue. Conversely, the anatomical region most frequently affected in females was found to be the buccal sulcus [19]. In contrast, Anwar *et al.*, showed that oral squamous cell carcinoma (OSCC) exhibited a prevalence twice as high in the buccal mucosa, accounting for 68.8% of cases [20]. Likewise, investigations carried out in 2018 by Mehdi *et al.*, and in 2019 by Akram *et al.*, reported that the buccal mucosa stood out as the most frequently involved site in individuals with oral cancers [21, 22]. In our research, the predominant observation was that most of the cases showed well differentiation, which was followed by moderate and poor differentiated cases. Consistent with our investigation Sarfaraz *et al.*, in 2020 reported that well-differentiated cases were the most commonly observed grade in oral squamous cell carcinoma (OSCC) cases [23]. Contrary to our findings, a study in 2019, Mehdi *et al.*, from Pakistan found an equal number of moderate and poor grade cases

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(n=16, 34%), with a slightly lower number (n=15, 32%) of well-differentiated cases [24]. Study from Pakistan Rehman *et al.*, similarly demonstrated that moderately differentiated cases were the most prevalent grade for oral squamous cell carcinoma (OSCC) [25]. Among the 53 participants, the majority of males (n=35) were identified as snuff users. On the contrary, among the total participants, 18 females were identified as snuff users. Our study's findings are consistent with those of Radika *et al.*, where they reported that 8% of men and 5% of women were users of snuff [26]. A majority of patients with a history of snuff use in our study exhibited well-differentiated OSCC (33%). In contrast, Sinha *et al.*, reported that females with lower education levels and from low socio-economic status backgrounds were more likely to use snuff [27]. In our research, a predominant occurrence of p16 positivity was noted in well-differentiated OSCC cases, followed by moderately differentiated and poorly differentiated OSCC cases. However, no statistically significant association was found between p16 expression and the grades of OSCC. These findings align with a study reported Agarwal *et al.*, which similarly reported p16 expression and OSCC grade levels do not significantly correlate [28]. In contrast to our findings, the majority of OSCC cases with p16 positive were moderate grade (79.5%), followed by well and poor grade OSCC, according to a study conducted by Naz *et al* [29]. The observed differences in findings might be attributed to the methodology employed by Naz *et al.*, who conducted PCR on all participants' samples and subsequently selected only those that were HPV positive. Additionally, variations in results could be influenced by differences in sample sizes between the two studies. In our study, a notable association was observed between the history of snuff use and p16 expression, indicating that snuff users exhibited higher p16 expression. This correlation may be attributed to the adverse effects of snuff on the immune system, rendering patients more susceptible to viral infections. The outcomes of our study align with those reported by Naz *et al.*, and Aguayo *et al.*, as both studies demonstrated no significant association between snuff use and the p16 expression [29, 30]. The findings of Trinh *et al.*, in 2021 from France further support our results, as they similarly demonstrated no statistical significance for snuff use and p16 positivity [31]. Contrary to our findings, Agarwal *et al.*, in 2021 reported a statistical significance for snuff use and the expression of p16, with a p-value of 0.012 [28]. Similarly, a study revealed a statistical significance for snuff use and the positivity of p16 [32].

CONCLUSIONS

In OSCC, majority of males uses snuff with most frequent site involved was tongue. The oral squamous cell carcinoma (OSCC) cases that were well-differentiated exhibited the highest expression of p16, followed by moderate and poorly differentiated OSCC cases. However, no significant association was observed between p16 expression and OSCC in snuff users.

Authors Contribution

Conceptualization: KA
Methodology: KA, NK, RA, SY
Formal analysis: SA, SN
Writing-review and editing: KA, NK

All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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REFERENCES

[1] Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A *et al.* Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: a Cancer Journal for Clinicians. 2021 May; 71(3): 209-49. doi: 10.3322/caac.21660.

[2] Zhang SZ, Xie L, Shang ZJ. Burden of oral cancer on the 10 most populous countries from 1990 to 2019: estimates from the global burden of disease study 2019. International Journal of Environmental Research and Public Health. 2022 Jan; 19(2): 875. doi: 10.3390/ijerph19020875.

[3] Zhang LW, Li J, Cong X, Hu XS, Li D, Wu LL *et al.* Incidence and mortality trends in oral and oropharyngeal cancers in China, 2005–2013. Cancer Epidemiology. 2018 Dec; 57: 120-6. doi: 10.1016/j.canep.2018.10.014.

[4] Baig S and Rubab ZA. Trends leading to oral cancer. Pakistan Journal of Medicine and Dentistry. 2013; 2(1): 30-2.

[5] Jiang S and Dong Y. Human papillomavirus and oral squamous cell carcinoma: A review of HPV-positive oral squamous cell carcinoma and possible strategies for future. Current Problems in Cancer. 2017 Sep; 41(5): 323-7. doi: 10.1016/j.currprobIcancer.2017.02.006.

[6] Chamoli A, Gosavi AS, Shirwadkar UP, Wangdale KV, Behera SK, Kurrey NK, *et al.* Overview of oral cavity squamous cell carcinoma: Risk factors, mechanisms, and diagnostics. Oral Oncology. 2021; 121: 105451. doi: 10.1016/j.oraloncology.2021.105451.

[7] Baig S, Zaman U, Lucky MH. Human papilloma virus 16/18: Fabricator of trouble in oral squamous cell carcinoma. International Journal of Infectious Diseases. 2018 Apr; 69: 115-9. doi: 10.1016/j.ijid.2018.02.003.

DOI: https://doi.org/10.54393/pjhs.v5i04.1369

[8] Nasib B, Khattak MT, Mahmood R, Hasnain S, Tariq SF, Hassan M. Expression of P16 in Head and Neck Squamous Cell Carcinoma. Pakistan Journal of Medical & Health Sciences. 2022 Jan; 16(01): 1072-76. doi: 10.53350/pjmhs221611072.

[9] Ford PJ and Rich AM. Tobacco use and oral health. Addiction. 2021 Dec; 116(12): 3531-40. doi: 10.1111/add.15513.

[10] Jiang X, Wu J, Wang J, Huang R. Tobacco and oral squamous cell carcinoma: A review of carcinogenic pathways. Tobacco Induced Diseases. 2019; 17. doi: 10.18332/tid/105844.

[11] Lepore S, Lettini G, Condelli V, Sisinni L, Piscazzi A, Simeon V *et al.* comparative gene expression profiling of tobacco-associated HPV-positive versus negative oral squamous carcinoma cell lines. International Journal of Medical Sciences. 2020; 17(1): 112. doi: 10.7150/ijms.35133.

[12] Mubeen K, Chandrashekhar H, Kavitha M, Nagarathna S. Effect of tobacco on oral-health an overview. Journal of Evolution of Medical and Dental Sciences. 2013 May; 2(20): 3523-35. doi: 10.14260/jemds/723.

[13] Khokhar RA, Asif M, Khadim T, Malik TM. Incidence of human papilloma virus in patients of oral squamous cell carcinoma in a subset of pakistani population: Human Papilloma Virus in Oral Squamous Cell Carcinoma. Pakistan Armed Forces Medical Journal. 2018 Aug; 68(4): 717-22.

[14] Mishra MK, Gupta S, Shivangi, Sharma M, Sehgal S. The repertoire of mutational signatures in tobacco- and non-tobacco-induced oral cancer. Clinical and Translational Oncology. 2023 Dec; 25(12): 3332-44. doi: 10.1007/s12094-023-03192-8.

[15] Magnes T, Wagner S, Kiem D, Weiss L, Rinnerthaler G, Greil R *et al.* Prognostic and predictive factors in advanced head and neck squamous cell carcinoma. International Journal of Molecular Sciences. 2021 May; 22(9): 4981. doi: 10.3390/ijms22094981.

[16] Mastutik G, Rahniayu A, Kurniasari N, Rahaju AS, Alia R. Correlation between the Expression of E6 HPV with p53 and p16INK4A at Cervical Adenocarcinoma and Cervical Normal. Indian Journal of Forensic Medicine & Toxicology. 2021; 15(1): 1827-34.

[17] AnitaBalan MD and Balram P. Assessment of p16 Tumour Suppressor Protein Expression in Oral Mucosa of Smokers and Paan Chewers using Immunohistochemistry. IOSR Journal of Dental and Medical Sciences. 2019 Nov; 18(11): 69-74. doi: 10.9790/0853-1811036974.

[18] Yosefof E, Hilly O, Stern S, Bachar G, Shpitzer T, Mizrachi A. Squamous cell carcinoma of the oral tongue: Distinct epidemiological profile disease. Head & Neck. 2020 Sep; 42(9): 2316-20. doi: 10.1002/hed.26177.

[19] Ehtesham H, Safdari R, Mansourian A, Tahmasebian S, Mohammadzadeh N, Ghazisaeedi M *et al.* Clinical decision support system, a potential solution for diagnostic accuracy improvement in oral squamous cell carcinoma: A systematic review. Journal of Oral Health and Oral Epidemiology. 2017 Dec; 6(4): 187-95.

[20] Anwar N, Pervez S, Chundriger Q, Awan S, Moatter T, Ali TS. Oral cancer: Clinicopathological features and associated risk factors in a high risk population presenting to a major tertiary care center in Pakistan. Plos One. 2020 Aug; 15(8): e0236359. doi: 10.1371/journal.pone.0236359.

[21] Goertzen C, Mahdi H, Laliberte C, Meirson T, Eymael D *et al.* Oral inflammation promotes oral squamous cell carcinoma invasion. Oncotarget. 2018 Jun; 9(49): 29047. doi: 10.18632/oncotarget.25540.

[22] Akram S, Shabbir A, Mirza T. Association of high risk human papilloma virus (HPV-16/18) with P16 protein in oral premalignant lesions and oral squamous cell carcinoma. The Pakistan Journal of Medicine and Dentistry. 2019; 9: 4-11. doi: 10.36283/PJMD8-4/002.

[23] Sarfaraz A, Rashid S, Irshad M, Khattak MT, Shabir H. Different tumour grading in smokeless tobacco (Naswar) user and non-user in squamous cell carcinoma. The Professional Medical Journal. 2020 May; 27(05): 1054-8. doi: 10.29309/TPMJ/2020.27.05.4278.

[24] Mehdi RF, Shaikh F, Baig S, Qureshi MF, Lakhani M, Zia I *et al.* Oral squamous cell carcinoma and tobacco use. Journal of Advances in Medicine and Medical Research. 2018; 28(9): 1-5. doi: 10.9734/JAMMR/2018/46385.

[26] Rehman A, Naeem S, Khan SZ, Ali K, Waris S, farooq su. Histopathological pattern of squamous cell carcinoma at various intraoral sites. Journal of Khyber College of Dentistry. 2019 Sep; 9(03): 47-51.

[27] Mohideen K, Krithika C, Jeddy N, Parveen S, Radhika T, Sankari SL. A Meta-Analysis in Assessing Oxidative Stress Using Malondialdehyde in Oral Submucous Fibrosis. European Journal of Dentistry. 2021 Oct; 15(04): 675-81. doi: 10.1055/s-0041-1728230.

[28] Sinha DN, Gupta PC, Kumar A, Bhartiya D, Agarwal N, Sharma S *et al.* The poorest of poor suffer the greatest burden from smokeless tobacco use: A study from 140 countries. Nicotine and Tobacco Research. 2018 Nov; 20(12): 1529-32. doi: 10.1093/ntr/ntx276.

[28] Agarwal VK, Sharma R, Gahlot GP, Arnav A. Clinical and histopathological correlation of p16 and p53 expression in oral cancer. Indian Journal of Surgical

DOI: https://doi.org/10.54393/pjhs.v5i04.1369

Oncology. 2021 Apr; 12(Suppl 1): 164-8. doi: 10.1007/s13193-020-01145-z.

[29] Naz F, Verma H, Tanveer N, Sudheer AK, Kakkar A, Tanwar P. Demographic profile of p16 immunopositive and HPV DNA PCR positive oral squamous cell carcinoma in a large cohort of Indian patients. Asian Pacific Journal of Cancer Prevention: APJCP. 2022 Feb; 23(2): 529. doi: 10.31557/APJCP.2022.23.2.529.

[30] Aguayo F, Muñoz JP, Perez-Dominguez F, Carrillo-Beltrán D, Oliva C, Calaf GM *et al.* High-risk human papillomavirus and tobacco smoke interactions in epithelial carcinogenesis. Cancers. 2020 Aug; 12(8): 2201. doi: 10.3390/cancers12082201.

[31] Trinh JM, Thomas J, Salleron J, Henrot P. Differences in clinical and imaging characteristics between p16-positive non-smokers and p16-positive smokers or p16-negative patients in oropharyngeal carcinoma. Scientific Reports. 2021 Feb; 11(1): 3314. doi: 10.1038/s41598-021-82999-3.

[32] Hossain T. Identification of socio-demographic and behavioral risk factors of oral cancer in rural and urban areas of Bangladesh. [Doctoral dissertation]. Bangladesh; East West University: 2017.