Immunoexpression of P16 and Ki 67 in oral squamous cell carcinoma: Association with clinicopathological parameters

Shadan Mohamad Omer [1]   **Payam Anwar Rashid** [2]

Background and Objectives: Oral squamous cell carcinoma (OSCC) is considered as a major health problem worldwide and has been associated with high recurrence rate and poor prognosis. Advances in understanding of OSCC have not improved the outcome in their management significantly. Many studies have focused on the roles of biomolecular markers in OSCC. The use of p16 and Ki67 as biomarkers of biological behavior of oral squamous cell carcinoma is controversial. This study aimed to determine immunoexpression of P16 and Ki67 in oral squamous cell carcinoma and to evaluate their association with various clinicopathological parameters.

Materials and Methods: Fifty cases of squamous cell carcinoma from different locations in the oral cavity were included in this cross sectional study. The cases were collected from Rizgary Teaching Hospital and Private Laboratories in Erbil city during a period of eight months from October 2018 to May 2019. The expression of p16 and Ki 67 were evaluated immunohistochemically; the findings were correlated with the age of the patients, gender, site of the tumor and grade of the tumor.

Result: A total of 50 patients with oral squamous cell carcinoma were enrolled in this study the age ranged from 33 to 89 years, with a mean age ± SD of (64.24 ±12.01) years and more than half (52.0%) of them were males. Lower lip was the most common site of the tumor followed by upper lip and tongue (42.0%, 26.0% and 18.0%, respectively). Histopathological findings of the tumor showed that (54.0%) of the patients had moderately differentiated squamous cell carcinoma. However, (84.0%) of the patients showed negative expression of P 16, while Ki 67 expression was positive among (76.0%) of them. No significant statistical association were found between immunoexpression of p16 and age, sex of patient, site of the tumor and grade of the tumor (P=0.67, P=0,095, P=0.696, P=0.454 respectively). No significant statistical association were found between immunoexpression of Ki67 and age, sex of patient, site of the tumor and grade of the tumor (P=0.637, P=0,411, P=0.353, P=1.00 respectively).

Conclusion: in relation to the results obtained in this study no significant association were found between P16 and Ki 67 immunoexpression in oral squamous cell carcinoma with clinicopathological parameters. Further researches have to be designed to better understand the role of p16 and Ki 67 in OSCC.

Keywords: oral squamous cell carcinoma, immunoexpression, P16, Ki67.

(1)   College of Dentistry– Hawler Medical University

(2)   College of Medicine– Hawler Medical University

Introduction

Oral cavity cancer is a malignancy which arises from the mucosal epithelium lining the oral cavity, the organs of the oral cavity and salivary glands (especially the minor salivary glands) which were located on the wall of the oral cavity.[1, 2] Oral cancer is the sixth most found cancer More than 90% of malignancy of the oral in the world with an estimated 500,000 new cases each year. Frequency and risk factors differ in different geographic regions. Oral cavity cancer represents 3-5 % of all human malignancy. Oral cavity cancer is a major health problem and associated with severe morbidity, the mortality rate was 120,000 deaths annually.[3-5]

cavity is squamous cell carcinoma. Despite many progress in diagnosis and therapy for oral squamous cell carcinoma, current modality still unable to detect precancerous lesions like leukoplakia and erythroplakia which is the reason that majority of patients come with advanced disease.[6] Furthermore, approximately 20-50% cases had unknown clinical lymph node metastases to the neck and primary tumor recurrence within one year after treatment with poor prognosis. Current consideration in determining oral cancer treatment decisions (lymph node metastasis, histopathological type and grade) were considered unsatisfactory.1 Therefore, biomolecular markers were considered important for diagnostic and prognostic indicators.[7-10] P16 is an inhibitor of cell division, result of cyclin subordinate kinase N2 (CDKN2) quality, situated on the 9p21 chromosome, the inactivation of the locus is an early occasion in oral carcinogenesis.[11-15] Concentrates that have explored the statement of certain proteins engaged with these biomolecular systems show the perpetual worry for the recognizable proof of biomarkers with oral squamous carcinomas prescient potential.[9,16]

P16 are cell cycle control proteins, which give the "capture" and apoptosis of malignant growth cells and stops their expansion by keeping up in a hypophosphorilate status of retinoblastoma protein.[10,17] At present, there is no accord on the planning of event and prognostic noteworthiness of variant articulation of this protein.[18-22] The evaluation about the utilization of p16 as biomarkers will help the early recognizable proof of patients at high risk or patients enduring high-grade harm, which without a doubt will add to building up a superior and prior treatment.[12,23-24]

Sub-atomic natural markers have been proposed to be of an incentive in the conclusion and prognostic assessment of precancerous injuries.[8,25,26]

The Ki67 antigen, which encodes two protein isoforms with atomic loads of 345 and 395 kDa, was initially recognized by Scholzer and Gerdes in the mid 1980s.[10,27] The Ki67 protein has a half-existence of just ~1–1.5 h. It is available during every single dynamic period of the phone cycle (G1, S, G2 and M), however is missing in resting cells (G0).[11,12] In later periods of mitosis (during anaphase and telophase), a sharp diminishing in Ki67 levels happens.[13,22] Articulation of the Ki67 protein (pKi67) is related with the proliferative movement of natural cell populaces in harmful tumors, enabling it to be utilized as a marker of tumor aggressiveness.[3,14] The prognostic estimation of pKi67 has been explored in various examinations with its potential as a dependable marker having been appeared in diseases of the bosom, delicate tissue, lung, prostate, cervix and focal sensory system.[9,15] Current order plans may require update where natural conduct and prognostic criticalness of these tumors is worried, as an expanding number of studies have proposed that Ki67 might be a significant factor in disease reviewing and prognostic assessment. It has been demonstrated that Ki67 immunohistochemical staining is a powerful strategy for evaluating the visualization in various tumor types.[8,16] Despite the fact that pKi67 is a key marker related with multiplying malignant growth cells and a poor guess, its maximum capacity in expanding expansion has not been assessed. Numerous studies have shown that Ki-67 is relevant in breast, lung and prostate cancer prognosis. Although the significance of Ki-67 in head and neck cancers has been discussed during the past 20 years, no study revealed this controversy in reliable manner.[17,20,27]

Materials and Methods:

After obtaining approval of study protocol from research ethic committee at College of Dentistry in Hawler Medical University, Fifty cases of squamous cell carcinoma from different locations in the oral cavity were included in this cross sectional study. The cases were collected from Rizgary Teaching Hospital and Private Laboratories in Erbil city during a period of eight months from October 2018 to May 2019. The data for each patient including age, gender, tumor location, and tumor grade were obtained from database of the pathology reports. The Haematoxylin and Eosin (H&E) staining sections of all cases were reviewed and two sections of 4 micrometers thickness were taken from each paraffin embedded tissue block for immunohistochemical stain-

ing of P16 and Ki67, the sections de-waxed for 15 minutes in xylene and rehydrated with ethanol. Slides were treated with 3% hydrogen peroxidase solution for 30 minutes at room temperature. After being washed three times with phosphate buffered saline, microwave antigen retrieval was performed in citrate buffer (pH 6) for 5 minutes. For immunohistochemical staining we used avidine-biotin-peroxidase complex procedure in the IHC analysis (DakoCytomation, Copenhagen, Denmark). Then the tissue was stained by Anti-CDKN2A/p16INK4a antibody at dilution of 1:250 and Ki-67 (Clone MIB-1, IgG1) at dilution 1:150, appropriate positive and negative controls were included in each run of IHC.

Immunohistochemical Scoring of P16 & Ki67. Positive expression of P16 gives cytoplasmic and nuclear staining, while positive expression of Ki67 gives nuclear staining only, positive cells were determined by examining 10 HPF with (400x), the extent of both immunostaining was assessed as follows 28: for intensity score; 0=none, 1= weak, 2= intermediate, 3= strong. For proportion score; 0=0%, 1= ≤ 10%, 2= 10-50 %, 3= > 50 %.
Total score= Proportion score X Intensity Score (range = 0-9), 0-4 -----›Negative, 6-9-----› Positive.

Statistical Analysis. Descriptive analysis was conducted to determine samples characteristics as well as proportion of variables using computerized software Statistical Package for Social Sciences (SPSS) program version 23. The associations between p16 and Ki67 with clinicopathological variables were evaluated by chi-square and Fisher's exact test.
P-value of <0.05 was considered significant.

Ethical Consideration:
This study was approved by ethical committee in college of dentistry, Hawler medical university, Erbil, Iraq.
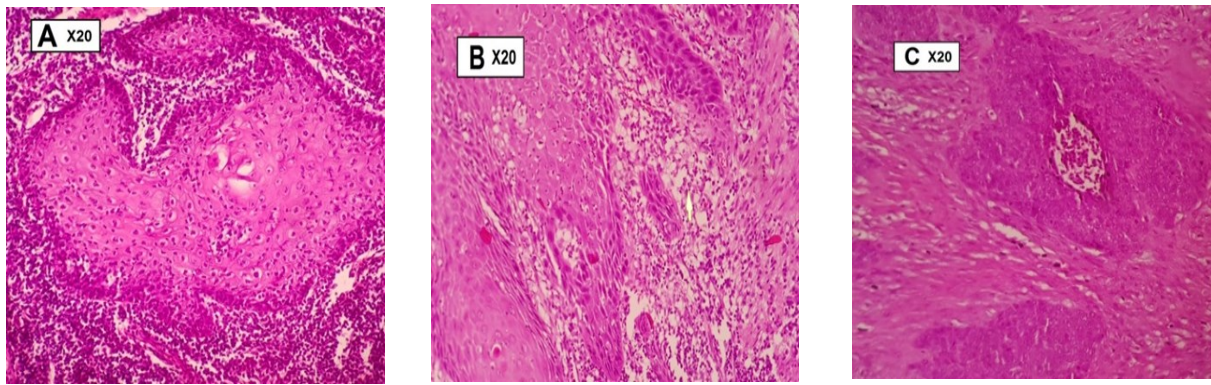
Results:
A total of 50 patients with oral squamous cell carcinoma were enrolled in the current study; their age ranged from 33 to 89 years, with a mean age ± SD of (64.24 ±12.01) years. Clinical profile of the patients showed that around two- thirds (72.0%) of the patients were aged 60 years old and more than half (52.0%) of them were males. Lower lip was the most common site of the tumor followed by upper lip and tongue (42.0%, 26.0% and 18.0%, respectively).
Histopathological findings of the tumor showed that more than half (54.0%) of the patients had moderately differentiated squamous cell carcinoma. However (84.0%) of the patients showed negative expression of P16, while Ki67 expression was positive among (76.0%) of them, as shown in (Table 1, Figures 1, 2 and 3).
Regarding association of P16 expression to

Table (1): Clinicopathological characteristics of the patients

Variables	No. & (%)
Age groups	
< 59 years	14 (28.0)
≥ 60 years	36 (72.0)
Sex	
Male	26 (52.0)
Female	24 (48.0)
Site of the tumor	
Upper lip	13 (26.0)
Lower lip	21 (42.0)
Tongue	9 (18.0)
Palate	2 (4.0)
Buccal mucosa	5 (10.0)
Grade	
Well differentiated	20 (40.0)
Moderately differentiated	27 (54.0)
Poorly differentiated	3 (6.0)
P 16 expression	
Positive	8 (16.0)
Negative	42 (84.0)
Ki 67 expression	
Positive	38 (76.0)
Negative	12 (24.0)
Total	50 (100.0)

A: Well differentiated.   **B: Moderately differentiated.**   **C: Poorly differentiated**

Figure 1: Grades of squamous cell carcinoma

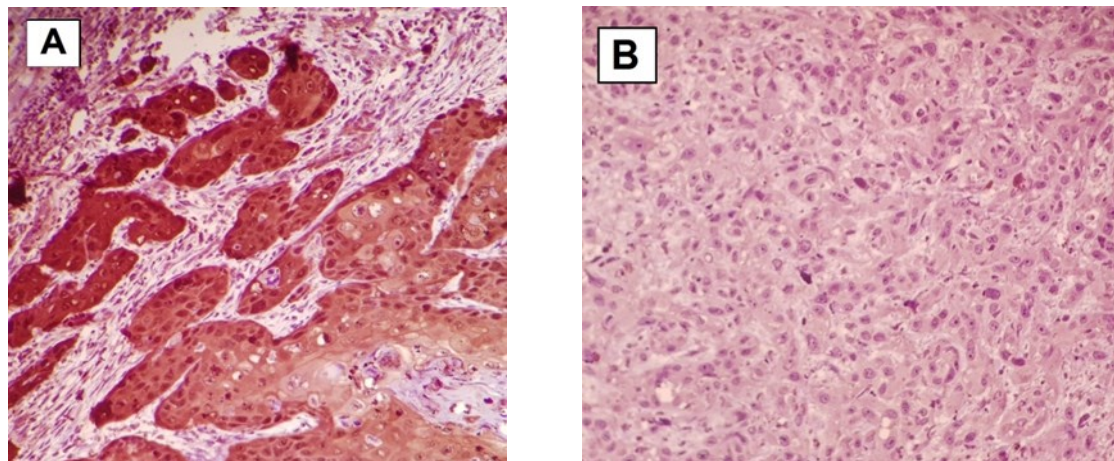**Figure 2: Positive and negative results of P16 expression in grade II OSCC**

(A: Positive   B: Negative)

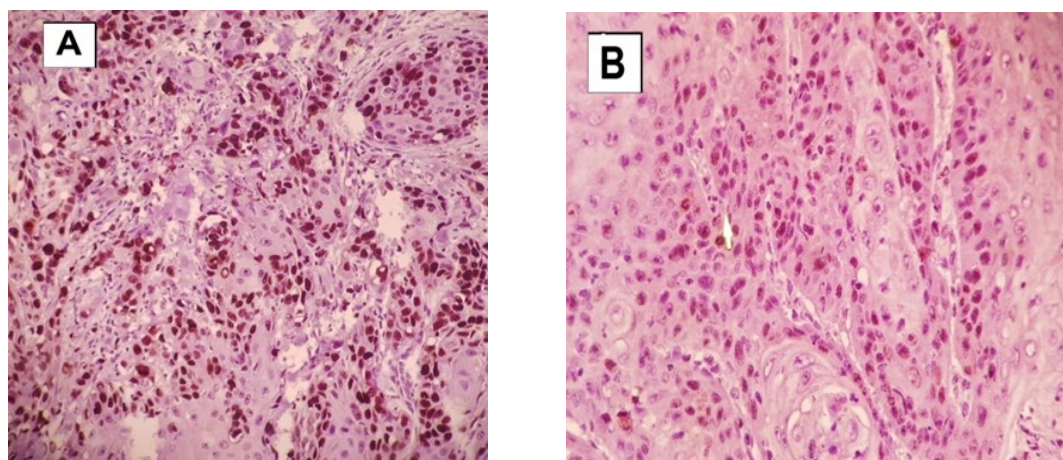**Figure 3: Positive and negative results of Ki 67 in grade II OSCC expression**

(A: Positive   B: Negative)

the age and gender of the patients, it was positive among (21.4%) of those aged less than 59 years, while it was positive among only (13.9%) of those ages 60 years and more with no significant statistical association (P= 0.67) as shown in (figure1).
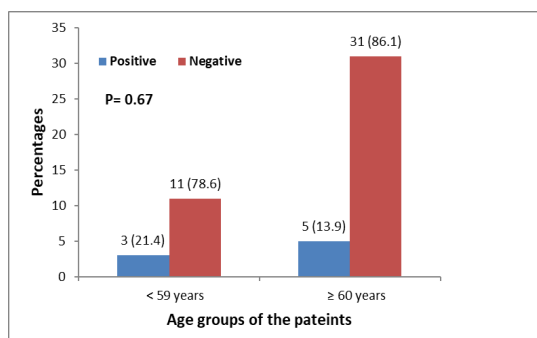
Also, there was no significant statistical association between the sex of the patients in relation to P16 expression (P= 0.095), where it was positive among (7.7%) of the males compared to (25.0%) among females, as shown in (figure 4).
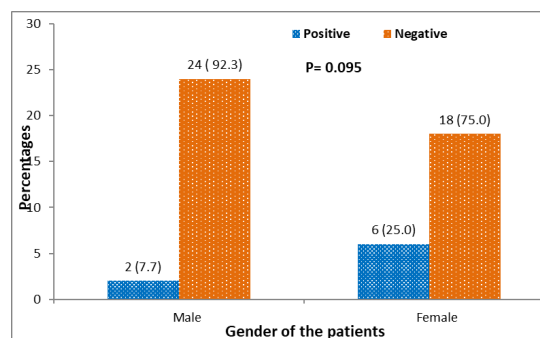
Concerning association of Ki 67 expression

Figure (4): Association of P16 expression with the age groups of the patients.

Figure (5): Association of P16 expression with the sex groups of the patients.

to the age and gender of the patients, it was positive among (71.4%) of those aged less than 59 years, while it was positive among (77.8%) of those ages 60 years and more with no significant statistical association (P= 0.637).

Also, there was no significant statistical association between the sex of the patients in

relation to Ki 67 expression (P= 0.411), where it was positive among (80.8%) of the males compared to (70.8.0%) among females, as shown in (Table 2).

About association of P 16 expression to the clinicopathological features of the patients, there was no significant statistical association between site of the tumor in relation to

Table (2): Association of Ki 67 expression to the age and sex of the patients.

Variables	Ki 67 expression No. & (%)		P value
	Positive	Negative	
Age groups			
< 59 years	10 (71.4)	4 (28.6)	0.637[*]
≥ 60 years	28 (77.8)	8 (22.2)	
Sex			
Male	21 (80.8)	5 (19.2)	0.411[*]
Female	17 (70.8)	7 (29.2)	
Total	**38 (76.0)**	**12 (24.0)**	

*P < 0.05 not significance of relationship

the P 16 expression (P= 0.696), where more P16 expression was reported among those having lower lip cancer (37.5%). The same is true about relation of P16 expression to the pathological grade of the tumor, where more positive results (62.5%) were reported among patients with moderately differentiat-ed squamous cell carcinoma (P=0.454), as shown in (Table 3).

Regarding association of Ki 67 expression to the clinicopathological features of the patients,(76%) of the patients had positive expression of Ki 67. Those with lower lip squamous cell carcinoma had more expres-

Table (3): Association of P 16 expression to the clinicopathological features

Variables	P 16 expression No. & (%)			P value **
	Positive	Negative	Total *	
Site of the tumor				
Upper lip	2 (25.0)	11 (26.2)	13 (26.0)	0.696***
Lower lip	3 (37.5)	18 (42.9)	21 (42.0)	
Tongue	1 (12.5)	8 (19.0)	9 (18.0)	
Palate	0 (0.0)	2 (4.8)	2 (4.0)	
Buccal mucosa	2 (25.0)	3 (7.1)	5 (10.0)	
Pathological grade				
Well differentiated	2 (25.0)	18 (42.9)	20 (40.0)	
Moderately differentiated	5 (62.5)	22 (52.3)	27 (54.0)	
Poorly differentiated	1 (12.5)	2 (4.8)	3 (6.0)	0.454***
Total	**8 (16.0)**	**42 (84.0)**	**50 (100.0)**	

*: Column percentage   **: Fisher's exact test   ***P < 0.05 no significant relationship

sion of Ki 67, followed by those having upper lip carcinoma (36.8% and 31.6%, respectively) , with no significant statistical association between Ki 67 expression and site of the tumor (P= 0.353).

Also, there was no significant statistical association between pathological grade of the patients to the Ki 67 expression (P= 1.0), where (55.3%) of those having moderately differentiated squamous cell carcinoma had positive expression, while (39.4%) of those having upper lip carcinoma had positive expression of Ki 67, as shown in (Table 4).

Discussion:

Oral squamous cell carcinomas, concerning the incidence and biological behavior sometimes unpredictable and still represent

Table (4): Association of Ki 67 expression to the clinicopathological features of the patients

Variables	Ki 67 expression No. & (%)			P value **
	Positive	Negative	Total*	
Site of the tumor				
Upper lip	12 (31.6)	1 (8.3)	13 (26.0)	0.353***
Lower lip	14 (36.8)	7 (58.3)	21 (42.0)	
Tongue	7 (18.4)	2 (16.7)	9 (18.0)	
Palate	2 (5.3)	0 (0.0)	2 (4.0)	
Buccal mucosa	3 (7.9)	2 (16.7)	5 (10.0)	
Pathological grade				
Well differentiated	15 (39.4)	5 (41.7)	20 (40.0)	
Moderately differentiated	21 (55.3)	6 (50.0)	27 (54.0)	1.0***
Poorly differentiated	2 (5.3)	1 (8.3)	3 (6.0)	
Total	**38 (76.0)**	**12 (24.0)**	**50 (100.0)**	

*: Column percentage   **: Fisher's exact test

***P < 0.05 not significance of relationship

a health problem worldwide. Although the lesions are easily accessible for clinical examination, oral squamous cell carcinoma prognosis can be difficult to assess in the context of location variability, risk factors, histopathological and molecular aspects involved in their appearance and progression. [28] The role of P16 and Ki 67 as biomarkers of behavior for malignant neoplasms and potentially malignant oral lesions remains controversial. The inactivation of P16 is an early event in oral carcinogenesis preceding the progression from premalignant to malignant oral lesions but the results which obtained from many studies were variable and some do not support this suggestion. [19]

In this study, the mean age ± SD was (64.24 ±12.01) years and the tendency for OSCC were more in age ≥ 60 years (72%) which was near to data obtained by Abrahao et al [1] and Ralli et al.,[32] while against that reported by Dragomir et al [28], Westra et al [29], Curado et al [30] and Ampur et al [31]. This difference may be due to different geographical areas and difference in sample size.

In the present study oral squamous cell carcinoma was more predominant in male (52%) which is in accordance to that reported by Abrahao et al [1], Dragomir et al[28] and Ralli et al [32] while the results reported by Ampur et al [31] showed female predominance (54.8%); he suggest that other factors such as smoking, alcohol, tobacco chewing, oral sex, and education, may be contributed to this difference in results. In this study the lower lip was the most common site of the tumor followed by upper lip and tongue (42.0%, 26.0% and 18.0%, respectively). Our results were comparable to that reported by Dragomir et al [28] while the results of other studies by Abrahao et al [1], Bradley et al [3], Linxweiler et al [9], Sundberg et al [26], Westra et al [29], Ampur et al [31], Ralli et al [32] and Fregonesi et al [33] showed variable location of the tumor this may be due to the role of risk factors such as smoking, alcohol, tobacco chewing, and abnormal sexual habit with their relation to HPV which were not analyzed in our study contributed to this difference in results.

Among the biomarkers with predictable potential for the prognosis of oral squamous carcinoma, P16 and Ki67 were intensively investigated in specialty literature with no

consensus in this direction.[31] P16 represents a negative regulator of cellular cycle that ensures the control of the cellular passage from phase G1 to phase S. Mitogenic stimuli, as well as the growth factors determine the activation of cyclin D, that binds and activates the cyclin-dependent kinases 4 and 6, resulting in retinoblastoma protein phosphorylation, following by the release of a transcription factor that ensures cell proliferation. [28] The role of p16 in the carcinogenesis and clinicopathology of OSCC has become a topic of research for the last decade. A study by Vairaktaris et al found a significant correlation between tumor progressions with tumor stage. [34]Thus, the investigation of p16 is useful in identifying dysplastic lesions, and the decrease of its immunoexpression is constituted as a predictive factor of neoplastic transformation of these lesions. However, there are still many differences between the results of studies on the role of p16 in OSCC.[28, 34]

In this study P16 showed positive immunoexpression in (16%) of cases while (84%) showed negative immunoexpression, our results were near to that reported by Abrahao et al [1], Dragomir et al [28], Ampur et al [31] and Ralli et al [32]. However, Yuen et al [35] in his study on the expression of p16 in OSCC found weak P16 expression in (48%) of cases. Differences between our study and Yuen et al is in terms of the number of samples since Yuen et al used 225 samples that may be more representative of the population there.

No significant association between p16 immunoexpression with age group, sex of patient and location of tumor were found in this study, our results is consistent with that reported by Dragomir et al [28], Ampur et al [31] and Ralli et al. [32]

Histological grade represent the degree of differentiation by applying a set of histological criteria. Grade is a strong and independent factor associated with distant metastasis in head and neck carcinomas. Thus, it adds important information to clinical and pathologic staging. It helps to identify patients at high risk for distant metastasis for whom an efficient systemic treatment is mandatory 32. In our study, maximum number of cases belonged to Grade II (54%). P16 immuoexpression had no significant association with

histological grade of the tumor, our results are in agreement with that reported by Yuen et al 35, and Dragomir et al 28. While studies by Ampur et al 31 , Ralli et al 32, Smith et al 36 and Muirhead et al 37,   observed that P16 immunoexpression was more likely to be detected with later stage and higher grade. They hypothesized that tumors that exhibited P16 expression had an effect on cell differentiation. These differences could also be attributed to difference in sample size, distribution of tumor site, different scoring criteria, and different type of antibodies used by different authors.

 The cell proliferation index was positively correlated with the degree of cell differentiation, and was higher in moderately and poorly differentiated cases. This finding corroborates the idea that the more undifferentiated the neoplasm, the poorer the control of the cell division process and the greater the proliferation 38.

 In the present study, Ki67 immunoexpression was identified in (76%) of cases although no significant association were found between Ki67 immunoexpression and age group, sex of patient, location of tumor and grade of tumor. The results are in accordance to data in the literature, Ki67 proving to be useful in assessing tumor aggressiveness 36-38.

The studies by Rodrigues et al 39 found no correlation between the immunoexpression of Ki-67 and prognostic value; however, it appears that Ki-67 over-expression associated with the idea of mitotic disarray, uncontrolled cell growth, and proliferation would be associated with prognostic factors and approach prioritization for the patients.

Conclusion:

 In relation to the results obtained in this study no significant association were found between P16 and Ki 67 immunoexpression in oral squamous cell carcinoma with clinicopathological parameters. Further researches with a larger sample size have to be designed to better understand the role of p16 and Ki 67 in OSCC.

Acknowledgments:

Conflicts of Interest:
The authors report no conflicts of interest.

Reference:

References

1. Abrahao AC, Bonelli BV, Nunes FD, Dias EP, Cabral MG. Immunohistochemical expression of p53, p16 and hTERT in oral squamous cell carcinoma and potentially malignant disorders. Braz Oral Res 2011;25(1):34-41.

2. Barnes L, Eveson J, Reichart P, Sidransky D. World Health Organization classification of tumours: pathology and genetics of head and neck tumours. 2005.

3. Bradley KT, Budnick SD, Logani S. Immunohistochemical detection of p16 INK4a in dysplastic lesions of the oral cavity. Modern pathology 2006;19(10):1310.

4. de Spíndula-Filho JV, Da Cruz AD, Oton-Leite AF, Batista AC, Leles CR, Alencar RdCG, et al. Oral squamous cell carcinoma versus oral verrucous carcinoma: an approach to cellular proliferation and negative relation to human papillomavirus (HPV). Tumor Biology 2011;32(2):409-16.

5. Fazeli Z, Pourhoseingholi MA, Pourhoseingholi A, Vahedi M, Zali MR. Mortality of oral cavity cancer in Iran. Asian Pac J Cancer Prev 2011;12(10).

6. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J of cancer 2010;127(12):2893-917.

7. Hopman AH, Smedts F, Dignef W, Ummelen M, Sonke G, Mravunac M, et al. Transition of high-grade cervical intraepithelial neoplasia to micro-invasive carcinoma is characterized by integration of HPV 16/18 and numerical chromosome abnormalities. J of the Pathol Society of Great Britain and Ireland. 2004;202(1):23-33.

8. Li Z, Wang Y, Yuan C, Zhu Y, Qiu J, Zhang W, et al. Oncogenic roles of Bmi1 and its therapeutic inhibition by histone deacetylase inhibitor in tongue cancer. Laboratory investigation 2014;94 (12):1431.

9. Linxweiler M, Bochen F, Wemmert S, Lerner C, Hasenfus A, Bohle RM, et al. Combination of p16INK4a/Ki67 immunocytology and hpv polymerase chain reaction for the noninvasive analysis of HPV involvement in head and neck cancer. Cancer cytopathology 2015;123(4):219-29.

10. Mikami T, Cheng J, Maruyama S, Kobayashi T, Funayama A, Yamazaki M, et al. Emergence of keratin 17 vs. loss of keratin 13: their reciprocal immunohistochemical profiles in oral carcinoma in situ. Oral oncology 2011;47(6):497-503.

11. Reuschenbach M, Seiz M, Doeberitz CvK, Vinokurova S, Duwe A, Ridder R, et al. Evaluation of cervical cone biopsies for coexpression of p16INK4a and Ki-67 in epithelial cells. Int J of cancer 2012;130(2):388-94.

12. Rosai J. Rosai and Ackerman's surgical pathology. 10th ed, volume one, USA, Elsevier, Mosby, 2011: 65.

13. Sritippho T, Chotjumlong P, Iamaroon A. Roles of human papillomaviruses and p16 in oral cancer. Asian Pac J Cancer Prev 2015;16(15):6193-200.

14. Warnakulasuriya S, Kovacevic T, Madden P, Coupland V, Sperandio M, Odell E, et al. Factors predicting malignant transformation in oral potentially malignant disorders among patients accrued over a 10-year period in South East England. J of oral pathology & medicine 2011;40 (9):677-83.

15. Yan J, Liu X-L, Han L-Z, Xiao G, Li N-L, Deng Y-N, et al. Relation between Ki-67, ER, PR, Her2/neu, p21, EGFR, and TOP II-α expression in invasive ductal breast cancer patients and correlations with prognosis. Asian Pac J Cancer Prev 2015;16 (2):823-9.

16. Yu L-L, Guo H-Q, Lei X-Q, Qin Y, Wu Z-N, Kang L-N, et al. p16/Ki-67 co-expression associates high risk human papillomavirus persistence and cervical histopathology: a 3-year cohort study in China. Oncotarget 2016;7(40):64810.

17. Akrish S, Ben-Izhak O, Sabo E, Rachmiel A. Oral squamous cell carcinoma associated with proliferative verrucous leukoplakia compared with conventional squamous cell carcinoma—a clinical, histologic and immunohistochemical study. Oral surgery, oral medicine, oral pathology and oral radiology 2015;119(3):318-25.

18. Shailaja G, Kumar JV, Baghirath P, Kumar U, Ashalata G, Krishna AB. Estimation of malignant transformation rate in cases of oral epithelial dysplasia and lichen planus using immunohistochemical expression of Ki-67, p53, BCL-2, and BAX markers. Dental research J 2015;12(3):235.

19. Gonzalez JCC, Cepeda LAG, Yanez SAB, Cornejo AD, Estevez ADM, Huerta ERL. p53 and p16 in oral epithelial dysplasia and oral squamous cell carcinoma: A study of 208 cases. Indian J of Pathology and Microbiology 2016;59(2):153.

20. Vallonthaiel AG, Singh MK, Dinda AK, Kakkar A, Thakar A, Das SN. Expression of cell cycle-associated proteins p53, pRb, p16, p27, and correlation with survival: a comparative study on oral squamous cell carcinoma and verrucous carcinoma. Applied Immunohistochemistry & Molecular Morphology 2016;24(3):193-200.

21. dos Santos Costa SF, Brennan PA, Gomez RS, Fregnani ER, Santos-Silva AR, Martins MD, et al. Molecular basis of oral squamous cell carcinoma in young patients: Is it any different from older patients? J of Oral Pathology & Medicine 2018;47 (6):541-6.

22. Rosa EA, Hurtado-Puerto AM, Falcão DP, Brietzke AP, de Almeida Prado Franceschi LE, Neto C, et

al. Oral lichen planus and malignant transformation: The role of p16, Ki-67, Bub-3 and SOX4 in assessing precancerous potential. Experimental and therapeutic medicine 2018;15(5):4157-66.

23. Sharada P, Swaminathan U, Nagamalini B, Vinodkumar K, Ashwini B, Lavanya V. A Semi-quantitative analysis of immunohistochemical expression of p63, Ki-67, Cyclin-D1, and p16 in common oral potentially malignant disorders and oral squamous cell carcinoma. J of Dr NTR University of Health Sciences 2018;7(2):120.

24. Beevi BH, Nayak SR, Peter CD, Haridas AK, Jacob L, Aboobakker A. Analysis of Ki-67 expression in oral premalignant lesions and normal oral mucosa: An immunohistochemical study. J of Pharmacy & Bioallied Sciences 2019;11(2):232.

25. Ciesielska U, Zatonski T, Nowinska K, Ratajczak-Wielgomas K, Grzegrzolka J, Piotrowska A, et al. Expression of cell cycle-related proteins p16, p27 and Ki-67 proliferating marker in laryngeal squamous cell carcinomas and in laryngeal papillomas. Anticancer research 2017;37(5):2407-15.

26. Sundberg J, Korytowska M, Burgos PM, Blomgren J, Blomstrand L, De Lara S, et al. Combined Testing of p16 Tumour-suppressor Protein and Human Papillomavirus in Patients With Oral Leukoplakia and Oral Squamous Cell Carcinoma. Anticancer research 2019;39(3):1293-300.

27. Maebayashi T, Ishibashi N, Aizawa T, Sakaguchi M, Saito T, Kawamori J, et al. Roles of Ki-67 and p16 as biomarkers for unknown primary head and neck squamous cell carcinoma. European Archives of Oto-Rhino-Laryngology 2019:1-9.

28. Dragomir LP, Simionescu C, Margaritescu C, Stepan A, Dragomir IM,Popescu MR. p53, p16 and Ki67 immunoexpression in oral squamous carcinomas. Rom J Morphol Embryol 2012;53:89‐93.

29. Westra HW. The Changing Face of Head and Neck Cancer in the 21st Century: The Impact of HPV on the Epidemiology and Pathology of Oral Cancer. Head and Neck Pathol 2009; 3:78–81.

30. Curado MP. dan Hashibe, M. Recent changes in the epidemiology of head and neck cancer. Curr.Opin.Oncol 2009;21, (3) 194-200.

31. Ampur OY, Sudarsa IW, Suryawisesa IB. Association of p16 expression with clinicopathological features of oral cavity squamous cell carcinoma patients in Bali. Bali Med J 2016; 5(3): 362-366.

32. Ralli M, Singh S, Yadav1 S.P.S., Sharma N. et al. Assessment and clinico-pathological correlation of p16 expression in head and neck squamous cell carcinoma. J of Cancer Research and Therapeutics 2016 ;12 (1):232-7.

33. Fregonesi P. A, Teresa D. B, Duarte R. A, et al. p16^INK4A Immunohistochemical Overexpression in Premalignant and Malignant Oral Lesions Infect-