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

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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.

Introduction
Oral 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 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. 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. Therefore, biomolecular markers were considered important for diagnostic and prognostic indicators. 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. 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. 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. At present, there is no accord on the planning of event and prognostic noteworthiness of variant articulation of this protein. 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. The Ki67 antigen 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). In later periods of mitosis (during anaphase and telophase), a sharp diminishing in Ki67 levels happens. 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. 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. 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.

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).

| 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) |

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Figure 1: Grades of squamous cell carcinoma

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

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 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 | P value |
|-----------|------------------|---------|
|           | No. & (%)        |         |
|           | 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 P16 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 differentiated squamous cell carcinoma (P=0.454), as shown in (Table 3).

Regarding association of Ki67 expression to the clinicopathological features of the patients, (76%) of the patients had positive expression of Ki67. Those with lower lip squamous cell carcinoma had more expression of Ki67, followed by those having upper lip carcinoma (36.8% and 31.6%, respectively), with no significant statistical association between Ki67 expression and site of the tumor (P=0.353).

Also, there was no significant statistical association between pathological grade of the patients to the Ki67 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 Ki67, as shown in (Table 4).

**Discussion:**

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

| Variables                        | P16 expression | P value ** |
|----------------------------------|----------------|-----------|
| Site of the tumor                |                |           |
| Upper lip                        | Positive       | Negative  | Total *  |
|                                  | 2 (25.0)       | 11 (26.2) | 13 (26.0) |
| 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              | Positive       | Negative  | Total *  |
|                                  | 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)   |

| Total                            | 8 (16.0)       | 42 (84.0) | 50 (100.0) |

| Variables                        | Ki67 expression | P value ** |
|----------------------------------|-----------------|-----------|
| Site of the tumor                |                 |           |
| Upper lip                        | Positive        | Negative  | Total*   |
|                                  | 12 (31.6)       | 1 (8.3)   | 13 (26.0) |
| 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              | Positive        | Negative  | Total*   |
|                                  | 15 (39.4)       | 5 (41.7)  | 20 (40.0) |
| Moderately differentiated        | 21 (55.3)       | 6 (50.0)  | 27 (54.0) |
| 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 no significant 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. 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.

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, Sundberg et al., while against that reported by Dragomir et al, Westra et al, Curado et al and Ampur et al. 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, Dragomir et al and Ralli et al while the results reported by Ampur et al 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 while the results of other studies by Abrahao et al, Bradley et al, Linxweiler et al, Sundberg et al, Westra et al, Ampur et al, Ralli et al and Fregonesi et al 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. 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. 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. 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.

In this study P16 showed positive immuno-expression in (16%) of cases while (84%) showed negative immunoexpression, our results were near to that reported by Abrahao et al, Dragomir et al., Ampur et al and Ralli et al. However, Yuen et al 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., Ampur et al and Ralli et al. 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. In our study, maximum number of cases belonged to Grade II (54%). P16 immunoexpression 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.

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