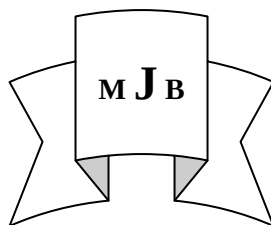


Expression of Ki-67 and p53 in Oral Squamous Epithelial Abnormalities

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Abstract

Objective: We intended to study the cellular proliferation by means of Ki-67 labeling index and the protein product overexpression of p53 in a series of oral intraepithelial lesions and squamous cell carcinoma, to evaluate the potential association between their histologic grades and the expression of the two markers. The expression of these markers on sections taken from hyperplastic and normal mucosal epithelium was also evaluated in order to determine whether the combination of p53 and Ki-67 over-expression could be used as a diagnostic aid in evaluating oral biopsies.

Materials and Methods: Archival biopsy specimens from 35 patients, with oral squamous cell carcinoma (19) and intraepithelial lesions (16) were retrieved from the histopathology departments of Erbil and Duhok cities, Iraq, between January and December 2010. From the same patients, 13 blocks with hyperplastic epithelium and 7 with normal mucosal epithelium were also retrieved. The study was done in Duhok Central Laboratory, Duhok, Iraq. Ki-67 labeling index and p53 overexpression was determined by immunohistochemistry on paraffin sections, using avidin-biotin technique.

Results: Overall, the staining patterns for Ki-67 antigen and p53 were similar. High Ki-67 and p53 overexpression was observed in 52.6% and 63.2% of carcinoma cases respectively, in 62.5% and 56.3% of intraepithelial lesions respectively, in 23.1% and 15.4% of hyperplastic epithelium respectively, and none in benign epithelium. Both high Ki-67- labeling index and p53 overexpression were significantly higher in carcinomas and intraepithelial lesions than in hyperplastic epithelium and were associated with the grade of both carcinomas and intraepithelial lesions.

Conclusions: Combination of p53 and Ki-67 overexpressions can be used as a specific marker for oral lesions that are probably at high risk for malignant transformation, their immunohistochemistry emerges as a clinically useful supplement for histopathological assessment of grading of oral squamous cell carcinoma and intraepithelial lesions.

Key words: Ki-67, p53, oral intraepithelial lesion, oral cancer

الخلاصة

الهدف: دراسة الانتشار الخلوي عن طريق مؤشر Ki-67 والتعبير المفرط للجين p53 في سلسلة من الآفات داخل ظهاري الفم وسرطان الخلايا الحرشفية، لتقييم مدى ارتباط محتمل بين درجاتهم النسيجية والمؤشرات الخلوية. كما تم تقييم التعبير عن هذه العلامات على المقاطع المأخوذة من مخاطية المفرطة التصنع والعادي من أجل تحديد ما إذا كان يمكن استخدام مزيج من p53، ki-67 والإفراط في التعبير كوسيلة مساعدة التشخيص في تقييم خزعات عن طريق الفم.

مواد وطرق: تم استرجاع المحفوظات لعينات الخزعة من ٣٥ مريض. الذين يعانون من سرطان الخلايا الحرشفية عن طريق الفم (١٩) والآفات داخل الظهاري (١٦) من امختبرات التشريح المرضي من مدينتي أربيل ودهوك ، ما بين يناير وديسمبر ٢٠١٠. من المريض نفسه، كما تم مراجعة ١٣ عينة من ظاهرة المفرطة التصنع و ٧ مع مخاطية طبيعية. ان الدراسة اجريت في المختبر دهوك المركزي ، دهوك، العراق. كان تقييم ki-67 مؤشر وضع العلامات و التعبير المفرط للجين p53 بالدراسة النسيجية المناعية على نماذج البارافين، وذلك باستخدام تقنية أفيدين-البيوتين.

النتائج: وعموما، كانت أنماط تطيخ للمستضد ki-67 وجين p53 متماثلة. لوحظ ارتفاع ki-67 وجين p53 التعبير المفرط للجين في ٥٢.٦٪ و ٦٣.٢٪ من حالات سرطان على التوالي، وفي ٦٢.٥٪ و ٥٦.٣٪ من الآفات داخل الظهاري على التوالي، وفي ٢٣.١٪ و ١٥.٤٪ على التوالي من ظاهرة المفرطة التصنع، وليس في الظهارة الحميدة. كان كل من ارتفاع ki-67 والتعبير المفرط للجين p53 أعلى بشكل ملحوظ في سرطان الخلايا الظهارية والآفات مما كانت عليه في ظاهرة المفرطة التصنع وترافقت مع درجة من كل من سرطان الخلايا الظهارية والآفات.

الاستنتاجات: يمكن استخدام مزيج من p53 و التعبير المفرط للجين ki-67 كعلامة محددة للآفات الفم التي هي على الأرجح الأكثر عرضة للتحويل للورم الخبيث. الدراسة النسيجية المناعية اظهرت اهمية من الناحية السريرية لتقييم المرضية في الدرجات لسرطان لخلايا الحرشفية للفم والخلايا الظهارية الآفات.

Introduction

Oral squamous cell carcinoma (SCC) is relatively uncommon but especially aggressive cancer. It is associated with a high rate of local recurrence and poor survival.[1] Like other cancers, oral SCC represents an accumulation of defects in the genes that encode key proteins associated with growth and development. This appears through a series of precancerous stages, manifested morphologically as epithelial dysplasia or intraepithelial lesions (IEL) in the sequence of dysplasia-carcinoma.[2-5] The increased proliferative activity observed in oral SCC compared to non-neoplastic hyperplasia illustrates a continuum between benign and malignant squamous epithelia.[6-8] Identification of the specific gene proteins and the sequence in which they appear in the normal, premalignant and malignant cells is necessary for the formulation of new treatment strategies, the development of early detection methods and the prediction of patient outcome.[4,9,10] p53, a tumor suppressor gene, is reported to be the most frequent target for genetic alterations leading to cancer. This gene

regulates cell proliferation and DNA repair by inhibiting the cell cycle at G1/S phase; loss of p53 function may therefore lead to aberrant cell kinetics.[4,11] Mutation of the p53 gene is thought to be an important component of oral carcinogenesis.[11] Immunoreactivity or overexpression of p53, an indicator of an abnormal accumulation of mutated p53, has been proposed as a reliable marker associated with oral carcinogenesis,[10-12] and a high percentage of oral squamous cell carcinomas show high levels of p53 expression.[5,6,10,12] Although in other kind of tumors, p53 overexpression is a late event, in oral cavity it can be observed in more initial phases.[5,13]

The human Ki-67 protein, which is strictly associated with cell proliferation, is expressed in all phases of the cell cycle excepting G0.[2,14] In oral mucosal lesions, the expression of Ki-67 has been reported to increase according to the proliferative activity and degree of epithelial dysplasia, suggesting that it is a good marker of cellular proliferation in premalignant and malignant lesions and is informed as a marker of the presence and severity of

oral IEL.[2,10,13,14] The Ki-67 labeling index (LI), i.e., the percentage of cells in a tissue staining for Ki-67 is a widely used marker of cell proliferation and can be used as an indicator to predict the condition as pre-malignant or malignant lesion.[10,12,13,15]

The present study is an attempt to extend the previous studies to elucidate the overexpression of cell proliferation protein (Ki-67) and the protein product p53 in a series of graded oral intraepithelial lesions (IEL) and untreated graded oral squamous cell carcinoma (SCC) at the invasive front, to evaluate the potential association between the expression of the two markers and the histologic grade of oral IEL and SCC. The relationship between the expression of these markers in hyperplastic epithelium and normal mucosal epithelium from the same patients was also evaluated in order to determine whether the combination of p53 and Ki-67 over-expression could be used as a diagnostic aid in evaluating oral mucosal biopsies.

Materials and Methods

In this retrospective study, a total of archival biopsy specimens were taken from 35 patients with histopathologically confirmed oral squamous cell carcinoma (19) and intraepithelial lesions (16). These cases were retrieved from various histopathology laboratories in Erbil and Duhok cities, Iraq, between January and December 2010. From the same patients, 13 blocks with hyperplastic epithelium and 7 with normal mucosal epithelium were also retrieved. The study was conducted in Duhok Central Laboratory, Duhok, Iraq. A representative block was selected for each case, for SCC, blocks showing invasive front were selected. Three serial (3-4-µm thick) sections

were cut from each case, one for hematoxylin and eosin, and other two were subjected to immunostaining for Ki-67 and p53. All histology slides were reviewed by two pathologists (Intisar S. Pity and Jalal A. Jalal) to confirm the diagnoses. Squamous cell carcinoma cases were graded as well differentiated (7), moderately differentiated (5) and poorly differentiated (7) while intraepithelial lesions were graded as low grade intraepithelial lesions (LGIL) (6) and high grade intraepithelial lesions (HGIL) (10) according to the WHO classification.^{16, 17}

Immunohistochemistry

The cell proliferation was studied by immunostaining for the cell cycle-dependent protein Ki-67 labelling index and the over expression of p53 protein was determined by immunohistochemistry. Sections were mounted on coated slides with polylysine and incubated overnight in 37 °C, then heated at 60 °C before staining. Sections were deparaffinized in xylene and rehydrated in graded alcohol, then transferred and rinsed twice with phosphate buffered saline (PBS, Dako Denmark A/S). Endogenous peroxidase was quenched by 3% hydrogen peroxide in methanol. After three times wash with PBS, epitopes were retrieved by heating sections in a pressure pot in sodium citrate buffer (pH 6.0) up to 3 minutes after boiling starts. After cooling, slides were washed three times with PBS and incubated overnight at 4°C with primary antibodies for Ki-67 using a mouse anti human monoclonal antibody (clone MIB-1 ready to use, Dako, Denmark A/S) and for p53 using a mouse monoclonal antibody against the p53 antigen (clone DO7, pre-diluted, Dako, Denmark A/S). After that, slides were rinsed gently three times with PBS and

incubated for 10 minutes with the biotinylated goat antipolyvalent antibody (Dako, Denmark A/S) at room temperature and an Envision Dual link system-HRP (ready to use, Dako, Denmark A/S) was used as the secondary antibody. Slides were again washed three times with PBS. Incubation with 3,3-diaminobenzidine tetra hydrochloride was performed for 5 minutes at room temperature as a substrate chromogen solution to produce a brown color. Finally, sections were counterstained with Mayer's haematoxylin, dehydrated and mounted. Appropriate positive control sections (high p53 expression breast cancer tissue for p53 and a lymph node with Burkitt's lymphoma for Ki-67) were processed in parallel. Specificity of staining was checked on negative control slides in which the primary specific antibodies were substituted by a buffer solution.

For Ki-67 and p53, a semiquantitative study was carried out by two independent pathologists. Expression of p53 was analyzed in the nucleus by assessing the percentage of marked cells and the staining intensity by counting 1,000 cancer cells in each sample (magnification, $\times 400$) and assessing the percentage of labelled cells. Staining for p53 was valued as positive (that is over expression) when there was $\geq 10\%$ nuclear staining. Ki-67 labelling index was performed by determining the labelling index (LI) of the marker in each case as described by Dudderidge et al.[18] Slides were evaluated at low-power magnification ($\times 100$) to identify the regions with the highest intensity of staining. From these selected areas, 3-5 fields at $\times 400$ magnification were captured with charge-coupled-device camera. Images were subsequently printed for quantitative analysis, which

was performed with the observer unaware of clinicopathological variables. Both positive and negative cells within the field were counted and any stromal or inflammatory cells were excluded. Cells with any degree of nuclear staining were scored positive. A minimum total of 500 cells were counted for each case. The LI was calculated using the following formula: $LI = \text{number of positive cells} / \text{total number of cells} \times 100$ as described and the index was calculated as a percentage. We adopted a 2-scale Ki-67 labelling index (LI): low ($<50\%$) and high (≥ 50) considering one cut-off point (50% Ki-67 LI).

Statistical Analyses

The descriptive statistics were analyzed with a Statistic Program for Social Sciences (SPSS) in Microsoft Excel. The Mann-Whitney *U*-test was used for appropriate *P* values. Data were recorded on specialized forms and all statistical tests were performed using SPSS version 16 for windows (SPSS Inc, Chicago, IL, USA) and Microsoft Excel (Realmond, W.A, USA) software. Descriptive analysis (e.g., mean, standard deviation, frequencies, percentage) were calculated and analysis was performed using the student's *t*-test and Fisher Exact *T*- Test. The chi-squared and Mann-Whitney tests were used to compare categorical variables. Analyses were performed with SPSS software (Statistical Package for the Social Sciences, version 15; SSPS Inc., Chicago, IL, USA). In all cases, we considered as significant with $p\text{-value} \leq 0.05$.

Results

The mean age of patients was 59.8 years (range: 37-78) and the male-female ratio was 1.7:1 (22 males, 13 females). The patterns of Ki-67 and p53

immunostaining, according to histology, are summarized in Table 1. It appears that the staining patterns for both markers are more or less similar and there was an obvious association between p53 positivity and high Ki-67 labelling index (LI) (Figure 1). Overall, the mean proliferative index, determined by Ki-67 LI, was absent or low (<50%) in all cases of normal epithelium (Figure 2). This index was also low in most hyperplastic epithelium (76.9%) (Figure 3), and LGIL (67.7%). On the other hand, Ki-67 LI was high ($\geq 50\%$) in HGIL (80%) (Figure 4). We found a significantly higher Ki-67 LI in HGIL than in benign epithelial proliferation ($p < 0.05$). The index was also significantly associated with the grade of intraepithelial lesions ($p < 0.05$). The Ki-67 LI was high in 52.6% of malignancy (Figure 5-7). It was significantly higher than that of hyperplastic epithelium ($p < 0.05$). Most well differentiated SCC revealed a low labelling index <50%, and the high labelling index was found mainly in high-grade tumors. There was a significant association between tumoral Ki-67 expression and its grade ($p < 0.05$). In addition to the varied percentages, the staining pattern also different; it was stronger and more diffuse in high grades IEL and SCC than their counterparts low grade lesions. No statistical significance was noted between Ki-67 staining of SCC and intraepithelial lesions ($p > 0.05$).

Similarly, the percentage of p53 immunostaining or overexpression

($\geq 10\%$ positively stained nuclei) increased with neoplastic severity in most tissues. It was negative in all normal epithelial samples (Figure 8). In hyperplastic epithelium, there was a patchy nuclear p53 staining distribution in 2 out of 13 (15.4%) cases (Figure 9). The p53-nuclear distribution was also noted as patchy in 2 out of 6 (33.3%) LGIL (Figure 10). A positive immunostaining for p53 was observed within HGIL in 7 of 10 cases (70%) (Figure 11). It was significantly higher in HGIL than hyperplastic epithelium ($p < 0.05$). Differences were also observed in the distribution of p53-positive cells between grades of intraepithelial lesions, with the development of compact p53-positive foci in HGIL. On the same line, p53 $\geq 10\%$ nuclear positivity was demonstrated in 63.2% of malignancy. The difference was significantly higher than hyperplastic epithelium ($p < 0.05$), and there was a significant increased p53 expression with tumor grade ($p < 0.05$). As Ki-67, the density of nuclear p53 expression also varied between tumor grades; moderate and poorly differentiated carcinomas displayed a denser and more diffuse p53-positivity than the well differentiated tumors (Figure 12-14), and statistically significant difference in percentage of p53 positive cells was not noted between intraepithelial lesions and invasive malignancy.

Table 1. Patterns of Ki-67 and p53 immunostaining according to histology

Histology (n)	High Ki-67 (%)	Low Ki-67 (%)	Positive P53 (%)	Negative P53 (%)
Normal epithelium (7)	0/7 (00)	7/7 (100)	0/7 (00)	7/7 (100)
Hyperplastic epithelium (13)	3/13 (23.1)	10/13 (76.9)	2/13 (15.4)	11/13 (84.6)
Intraepithelial lesion (16)*	10/16 (62.5)	6 (37.5)	9/16 (56.3)	7/16 (43.7)
LGIL (6)	2/6 (33.3)	4/6 (67.7)	2/6 (33.3)	4/6 (67.7)
HGIL (10) *	8/10 (80) *	2/10 (20)	7/10 (70)*	3/10 (30)
Squamous cell carcinoma (19)*	10/19 (52.6) *	9/19 (47.4)	12/19 (63.2)*	7/19 (36.8)
Well differentiated SCC (7)	1/7 (14.3)	6/7 (85.7)	2/7 (28.6)	5/7 (71.4)
Moderately differentiated SCC(5)	3/5 (60)	2/5 (40)	4/5 (80)	1/5 (20)
Poorly differentiated SCC (7)	6/7 (85.7) *	1/7 (14.3)	6/7 (85.7) *	1/7 (14.3)

*P-value ≤ 0.05

LGIL: Low grade intraepithelial lesion, HGIL: High grade intraepithelial lesion, SCC: Squamous cell carcinoma

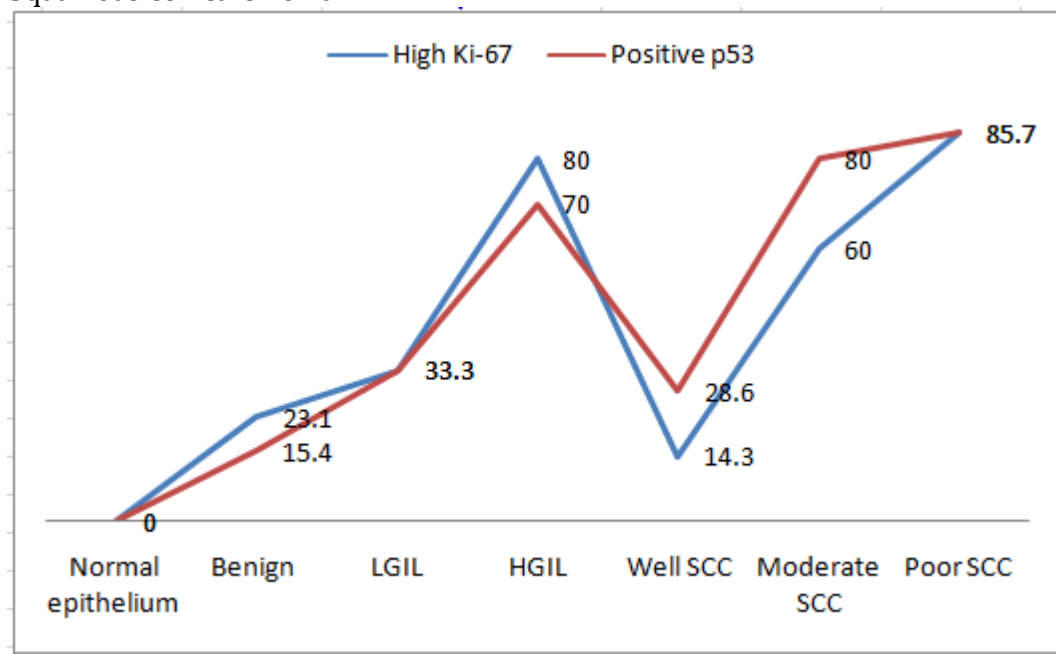
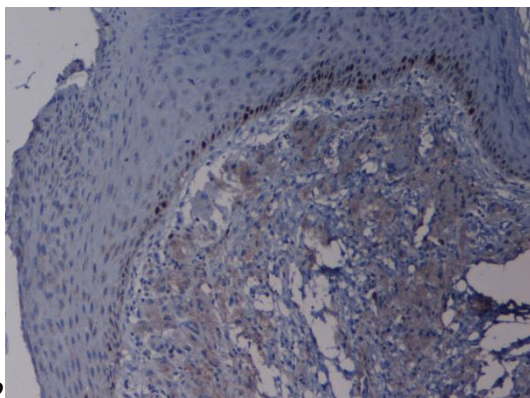
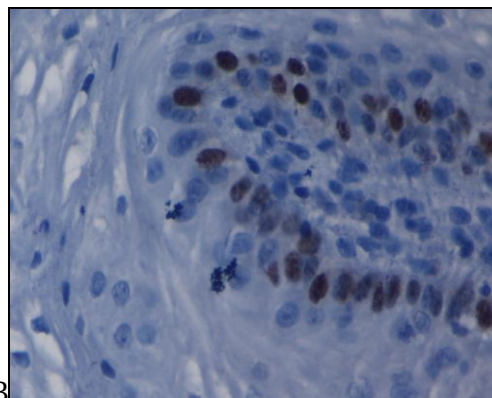


Figure 1. Patterns of high Ki-67 (blue) and positive p53 (red) within the different histology



2



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Figure 2. Low Ki-67 expression in benign oral epithelial proliferation.(x100)

Figure 3. Low Ki-67 expression in benign oral epithelial proliferation (hyperplasia) (x400)

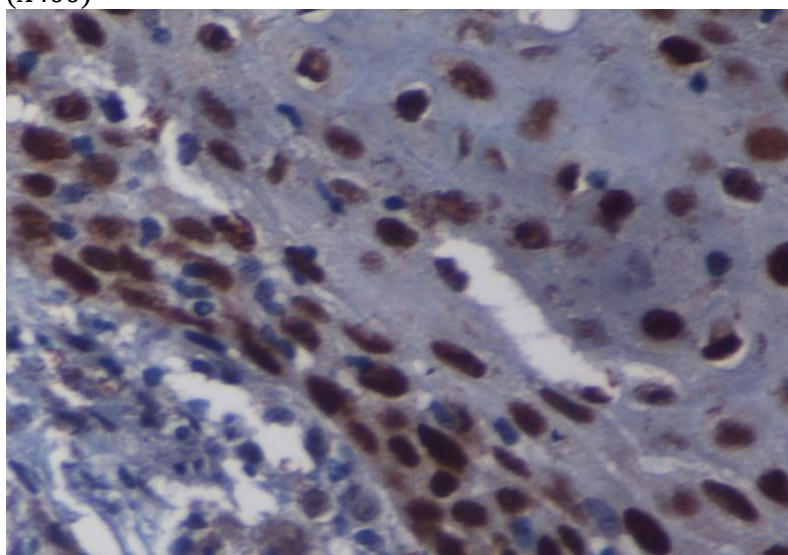


Figure 4. High Ki-67 expression in high grade intraepithelial lesion (x400).

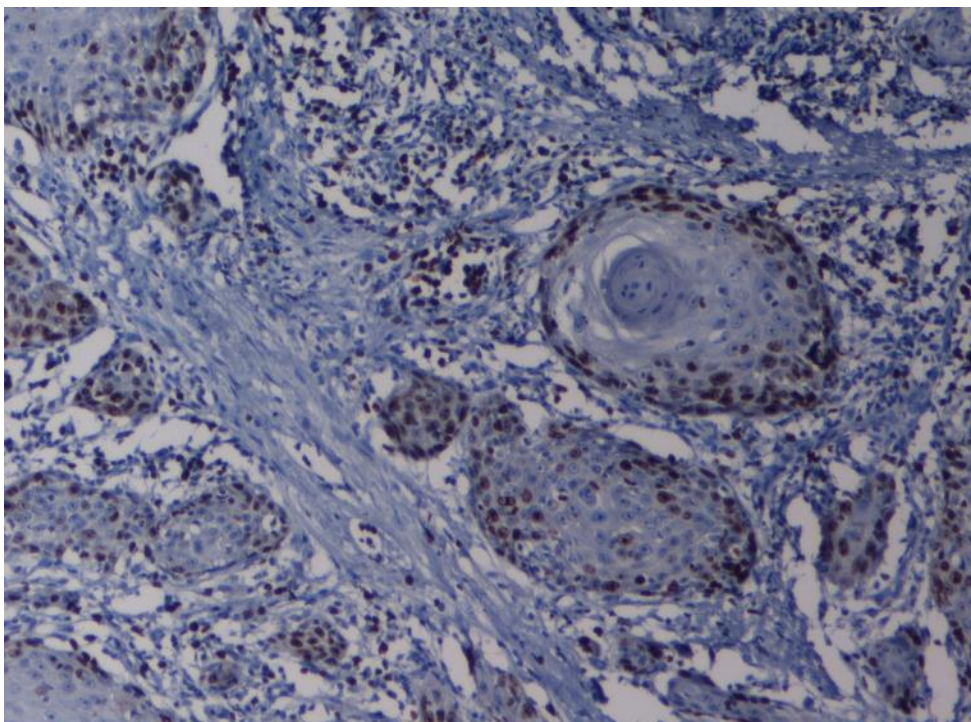


Figure 5. Ki-67 expression in the infiltrative margin of well differentiated squamous cell carcinoma (X 400)

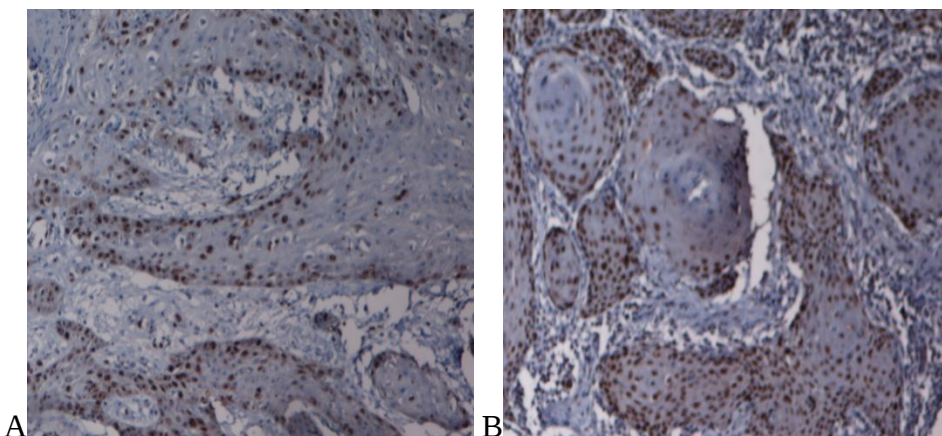


Figure 6. Ki-67 expression in in the infiltrative margin of moderately differentiated oral squamous cell carcinoma (A: low, B: High index) (x100)

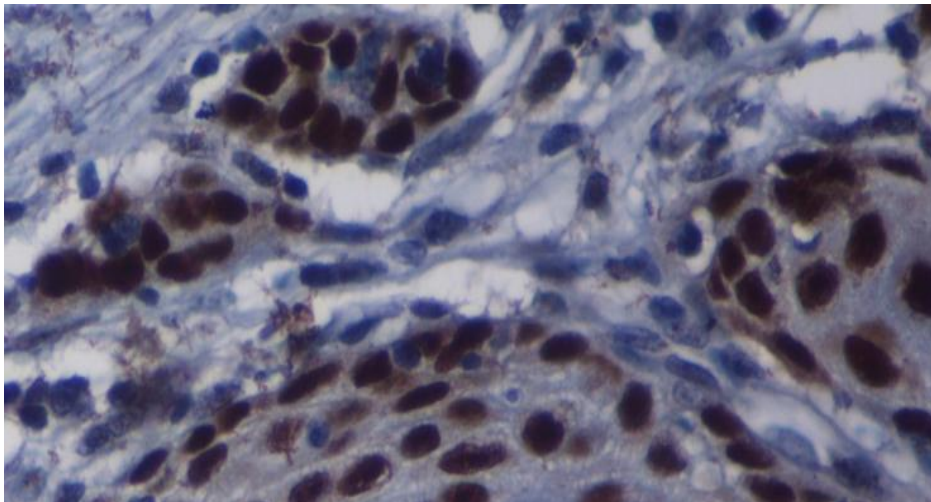


Figure 7. Ki-67 expression in the infiltrative margin of poorly differentiated oral squamous cell carcinoma (x400).

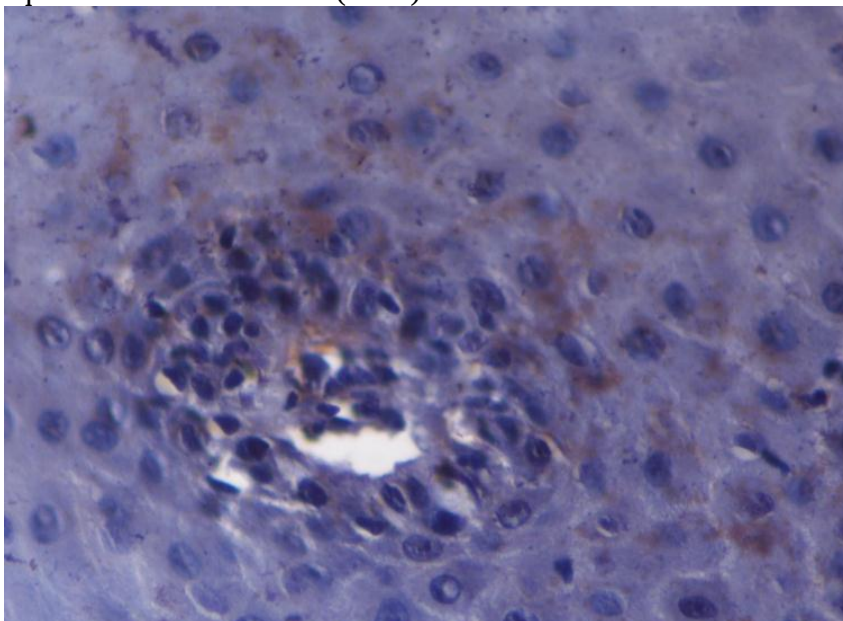


Figure 8. Negative p53 expression in normal oral epithelium (x100)

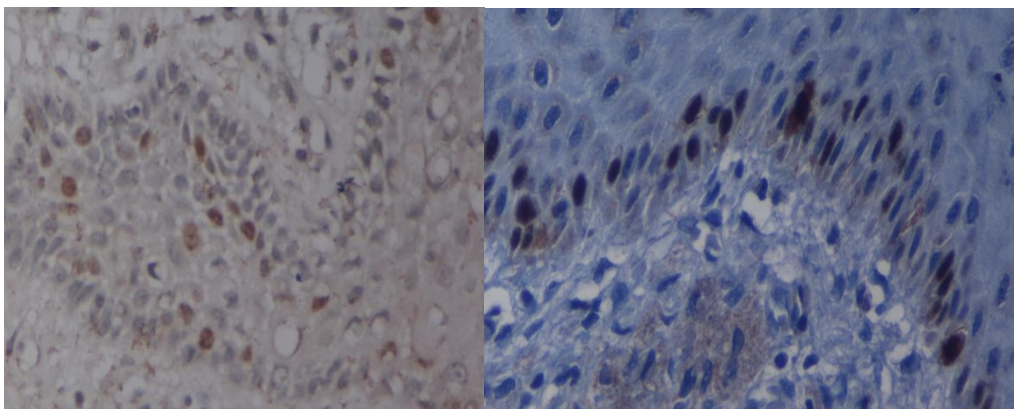


Figure 9. Patchy p53 expression in benign oral epithelial hyperplasia (x100)

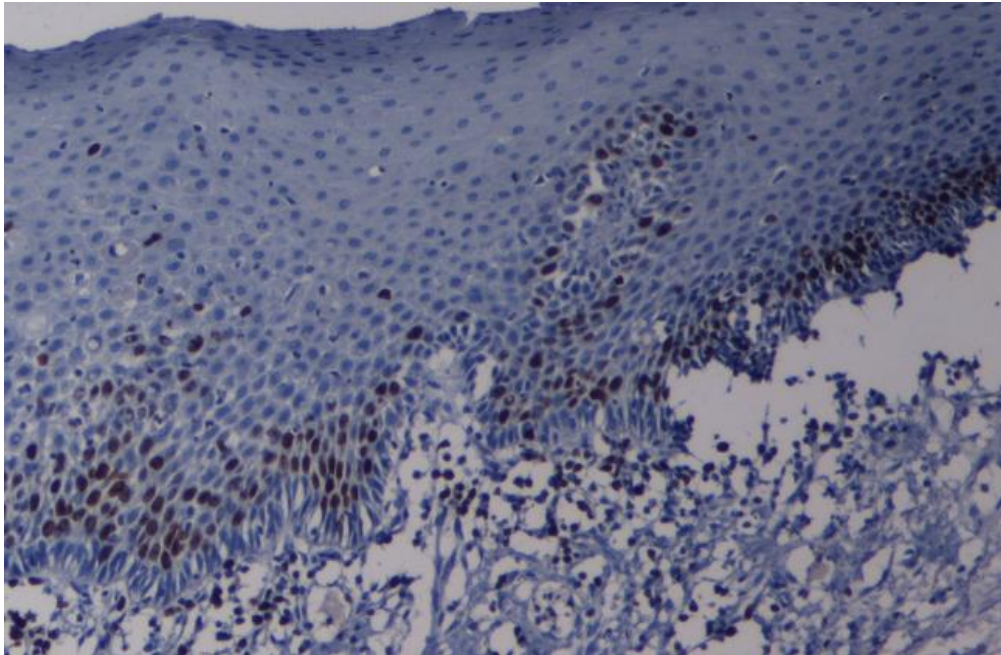


Figure 10. Patchy p53 expression in low grade oral intraepithelial lesion (x100).

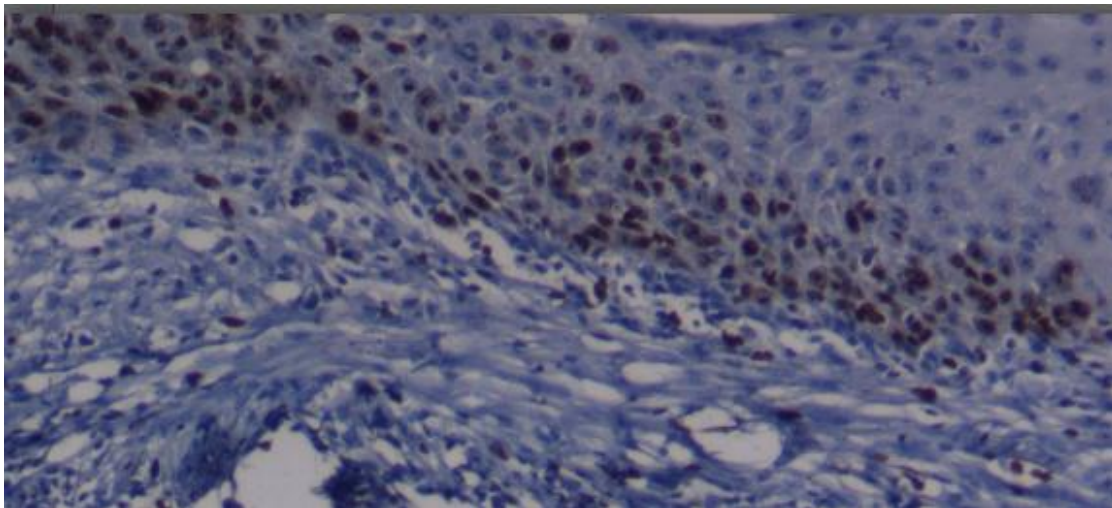


Figure 11. Positive p53 expression in high grade oral intraepithelial lesion (x100).

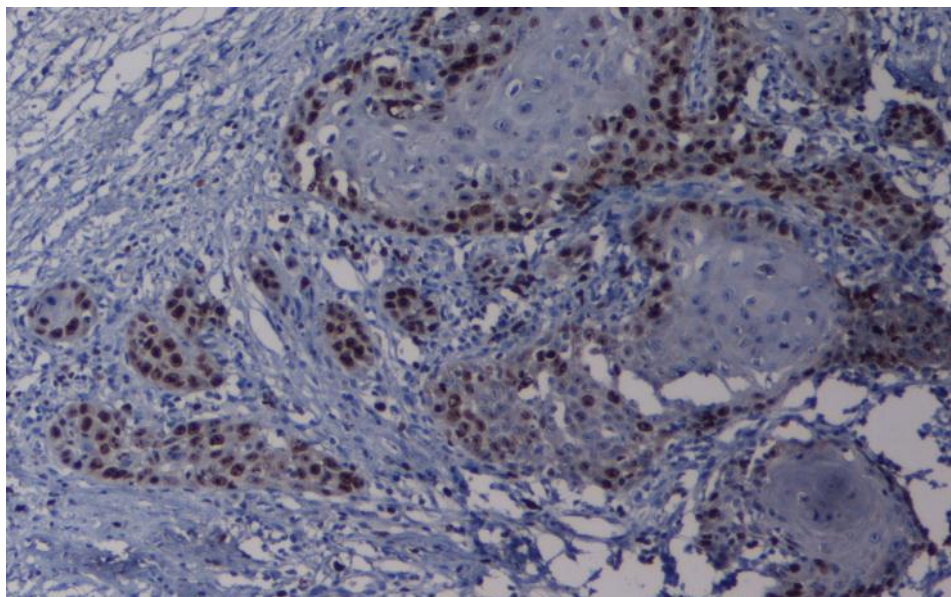


Figure 12. p53 expression in the infiltrative margin of well differentiated oral squamous cell carcinoma (x100).

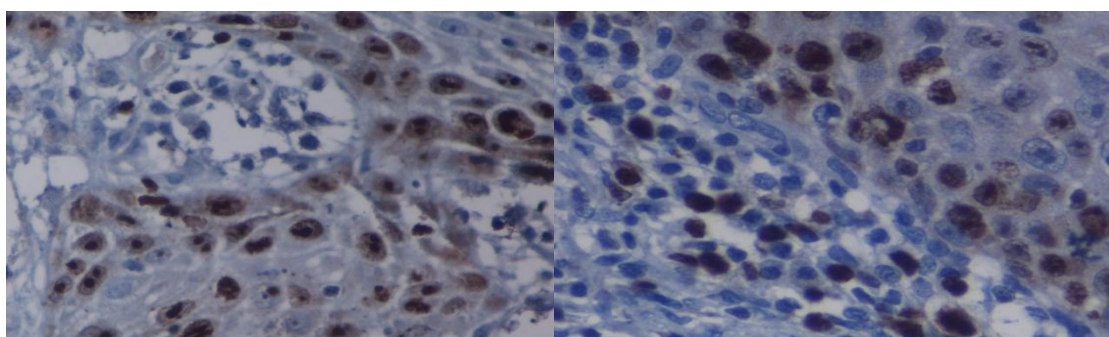


Figure 13. p53 expression in the infiltrative margin of moderately differentiated oral squamous cell carcinoma (x400)

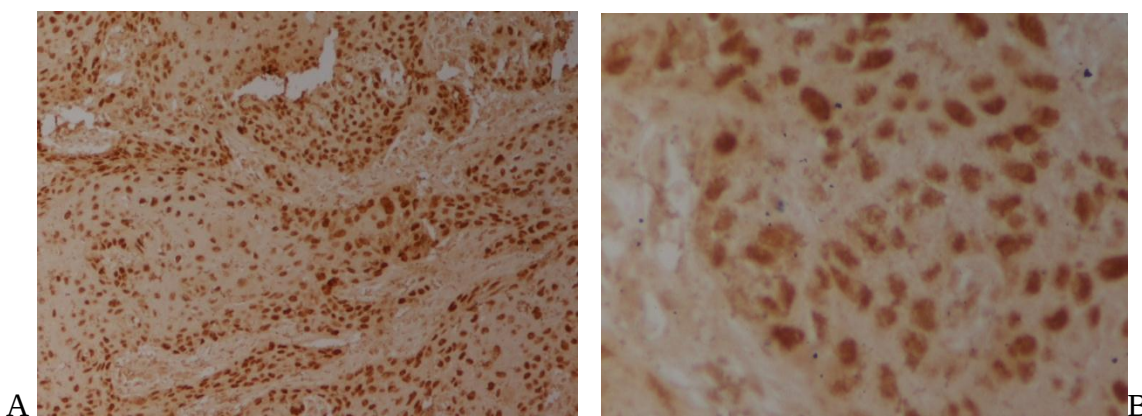


Figure 14. p53 in the infiltrative margin of poorly differentiated oral squamous cell carcinoma (A , B, x400)

Discussion

In the present study, we evaluated Ki-67 and p53 nuclear overexpression in oral biopsy specimens from 35 patients with intraepithelial lesions (IEL) and squamous cell carcinoma (SCC). p53 overexpression was found in 56.3% of IEL and 63.2% of SCC. These results fall within the range of values reported in the literature (11 to 90%).[7,10,15,19,20] The wide range values may be due to the substantial differences in detection techniques applied, the differences in clones of p53 used for immunohistochemical studies, oral hygiene, nutritional as well as the varied oral habits practiced in different geographical regions and races.[19-21] The p53 clone we used, DO7, has been used thoroughly in the study of p53 alterations in different organs and their results are broadly contrasted.[20,21] In our study, Ki-67 LI was high in 62.5% of IEL and in 52.6% of SCC. The results suggest that cells in oral SCC and IEL are in a high proliferation state as demonstrated by the biggest difference in values from hyperplastic epithelium to intraepithelial lesions and invasive SCC. This finding was also observed by other authors.[10,12-14] Co-expression and association between p53 and Ki-67 in oral IEL and SCC were demonstrated in our series, suggesting that alterations in the p53 protein might lead to increased cell proliferation. Both markers were significantly high in IEL and SCC when compared to hyperplastic epithelium. In accordance with our study, a similar panel was composed to separate SCC and dysplasia from hyperplastic epithelium of the oral cavity and larynx,[6,10,12,19-23] they noticed that p53 and Ki-67 overexpression have been suggested to be reliable indicators for SCC development. Coexpression of

these markers might be also helpful in distinguishing invasive carcinoma from reactive epithelial hyperplasia.[19,20] Mutation of p53 and hyperproliferation are thought to be an early event in oral carcinogenesis.[5,13] Dolcetti et al in his study on laryngeal neoplasia, suggested that the detection of p53 immunostaining in pre-invasive areas as well as in pre-neoplastic lesions was a very early event in laryngeal SCC.[23] The invasive front of SCC was selected for protein evaluation, because this is considered to be more relevant for the understanding of the cell-cycle kinetics of cells involved with invasion into the adjacent tissues.[8]

In our series, we also observed an increased Ki-67 and p53 expression, as a function of grade of histopathologic abnormalities in oral IEL and SCC. The highest levels of Ki-67 LI and p53 belonged to high grades IEL (80%, 70%) respectively and poorly differentiated SCC (85.7% each) respectively. The staining pattern was also different. In low grade intraepithelial lesions, the distribution of Ki-67 and p53 stained nuclei was patchy, just in contrast to high grade lesions in which the staining distribution was compact and diffuse. Likewise, in well differentiated SCC the number and distribution of the stained nuclear were obviously less marked than in poorly differentiated SCC. The increased cellular proliferation and p53 over expression were associated with more advanced lesions and that the distribution of proliferating cells in tissue may tell more about the regulatory mechanism that become dysfunctional during multistep process of carcinogenesis. This finding was also observed by other authors who found that the more differentiated the epithelium is the smaller positivity they

found and in those poorly differentiated epithelia, nearly all strata were positive for these markers. They also noted that this expression increased in a marked way, as there was advancing in the progression of dysplasia grade from oral lesions, with significant differences in the cellular proliferation between normal epithelium and mild and moderate dysplasias; larger differences were present for in situ and microinvasive carcinomas, suggesting that they are excellent markers of the presence and severity of IEL and SCC. [7,8,10,13,15,22,24]

Although these results need to be confirmed by other similar studies, Ki-67 LI and p53 expression showed no significant difference between the IEL and SCC groups. These findings could suggest that cells in IEL as those in SCC have an increased number of cells licensed to proliferate. In benign lesions, p53 positivity was of limited diagnostic utility; the marker was present in 2 of 13 (15.4%) benign epithelial proliferation. The low Ki-67 expression in epithelial cells in normal and non-neoplastic oral mucosa in this study, suggests that most epithelial cells in these mucosae are in the G0 and G0-G1 transition phases. Only about 23% of cells of non-neoplastic oral mucosa are actually in the cell cycle (demonstrated by Ki-67) suggesting that these tissue compartments have a low and controlled proliferation rate but with a continuous proliferative capacity. Low LIs in normal tissues have also been observed by other authors for Ki-67 in oral mucosa,[10] larynx,[22] and prostate tissues.[25] Complete absence of Ki-67 in some cases of normal epithelium suggests, according to the authors, that the proliferating cells could be stem cells that may pass through a prolonged cell

cycle. I.e. these cells are in a temporary G0 state.[4] Usually expression of p53 protein is very low in normal cells and under normal conditions it prevents the propagation of genetically-damaged cells.[4,11]

Conclusions

This study confirms and extends previous findings that the patterns of p53 and Ki-67 overexpression are similar and predominant in oral IEL and SCC; they reached their maximum values in HGIL and poorly differentiated SCC. Combination of p53 and Ki-67 overexpressions can be used as a specific marker for oral lesions that are probably at high risk for malignant transformation, their immunohistochemistry emerges as a clinically useful supplement for histopathological assessment of grading of oral squamous cell carcinoma and intraepithelial lesions.

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