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Analysis of KI-67 antigen in human Oral Squamous Cell Carcinoma – An immunohistochemical study

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Abstract:

Background: Ki-67 is a specific marker of proliferating cells and is useful in tumor diagnosis and prognosis. Our study aimed at demonstrating the proliferating tumor cells in randomly selected high power fields using Ki-67 immunostaining and relating its expression with the different histological grades of human oral squamous cell carcinoma (OSCC). *Methodology:* The study group comprised of 90 diagnosed cases of OSCC (33- well differentiated, 27- moderately differentiated, and 30 - poorly differentiated). Modified Broder's histological grading system was employed. The sections were stained with an antibody directed against the Ki-67 antigen using an advanced polymer staining system. In each case, the number of positive cells regardless of the staining intensity were estimated in five random high power fields (400X) using the image analyzer software. The Ki-67 labeling index (LI) was expressed as the number of positive nuclei / mm^2. Statistical analysis used were ANOVA and multiple comparisons test (Post-Hoc test) using the Bonferroni method. *Results:* A general increasing trend in the mean Ki-67 LI with higher Broder's grade was noted. However, a

statistically significant difference was obtained only between Ki-67 LI of well and poorly differentiated OSCC. Ki-67 LI of moderately differentiated OSCC did not have statistically significant difference with either well or poorly differentiated OSCC. *Conclusion:* We conclude that the tumor cell proliferation as measured by Ki-67 LI at randomly selected fields has a positive association with the histologic grading in human OSCC.

Key words: Cell proliferation; Human oral squamous cell carcinoma; Invasive tumor front; Ki-67 antigen; Randomly selected fields.

Introduction:

Cell proliferation is a biological process of vital importance and this control is lost in cancer.[1] Therefore, the knowledge of cellular proteins that control cell proliferation is essential for understanding tumor biology.[2], [3] Ki-67 antigen is a specific marker of proliferating cells.[4] Studies have shown a highly significant correlation between Ki-67 staining and the malignancy degree, and a marked variation within different tumor grades, indicating that Ki-67 staining is useful in tumor diagnosis and prognosis.[5] [6]

Various investigators have studied the Ki-67 expression at the invasive tumor front and also at the center of the tumor sections and have proved that Ki-67 LI at the invasive front is superior for prognostic purposes. Our effort was to quantify the Ki-67 positive cells in randomly selected fields, so as to facilitate the study of proliferative cells in incision biopsy specimens, in PD OSCC and in extensive tumors where obtaining the invasive tumor front is generally not possible.

Thus, our study aimed at demonstrating the proliferating tumor cells in randomly selected fields using Ki-67 immunostaining and relating its expression with the different histological grades of human OSCC.

Methodology:

This retrospective study was carried out on formalin fixed paraffin embedded tissue sections obtained from diagnosed cases of human OSCC. The study group comprised of 90 cases of OSCC which were histologically graded based on the Modified Broder's grading system. 33 cases were diagnosed as well- differentiated (WD OSCC), 27 cases as moderately differentiated (MD OSCC) and 30 cases as poorly differentiated (PD OSCC).

Only primary tumors originating from the oral cavity proper (tongue, floor of the mouth, palate, gingiva, alveolar, buccal, labial mucosa, vestibule and retromolar area) were included in the study. Secondary or metastatic tumors, tumors arising from vermilion border of lip and pharyngeal area and histological variants of OSCC were excluded.

The sections were stained with an antibody directed against the Ki-67 antigen using an advanced polymer staining system (BioGenex, USA). The staining was performed according to the immunohistochemical staining protocol. Presence of brown precipitate at the site of target antigen (nucleus) was indicative of positive immunoreactivity. Tissue sections of malignant brain tumor (Glioblastoma) were

taken as the positive control in our study. In each case, five random fields of the section were selected and those that were overlapping were carefully eliminated. These fields were photographed under 400X high power magnification using a 3 chip digital camera attached to a Carl Zeiss trinocular microscope with a 40X objective lens. The images were classified, transferred and stored in the computer [Figures 1], [Figure 2], [Figure 3].

The number of Ki-67 positive cells was counted in the photomicrographs of the five random high power fields in each case using the image analyzer software: Image-Pro-Express (Media Cybernetics, USA).The analysis of Ki-67 positive cells was done using a single parameter namely, manual tag for the number of Ki-67 positive cells. Iamaroon et al (2004)[7] have recommended counting of the positive cells irrespective of their staining intensity, and have also standardized the area covered by 400X high power field as equal to 0.1 mm^2.The above mentioned criteria were implemented. The Ki-67 LI was expressed as the number of positive nuclei / mm^2. The data of each case were noted and submitted for statistical analysis.

Results:

Six cases of WD OSCC did not stain despite the control being positive on repeated occasions. Hence they were excluded from the statistical analysis on the assumption that there was some technical error in the processing of the tissues. The final study sample consisted of 27 cases of WD OSCC, 27 cases of MD OSCC and 30 cases of PD OSCC. The mean Ki-67 LI for all the three groups was obtained [Table 1], [Graph 1] and analyzed using ANOVA [Table- 2] and multiple comparisons test (Post-Hoc test) using the Bonferroni method [Table 3]. The cell proliferation as measured by the Ki-67 LI at randomly selected fields increased with increasing Broder's grade (decreasing degree of differentiation). Among the three grades of OSCC studied, the least proliferative tumor was the WD OSCC followed by MD OSCC and the PD OSCC was the most proliferative tumor.

As described in Table-1, PD OSCC showed the highest mean Ki-67 LI of 1243.40 $\pm$ 810.77, followed by MD OSCC with a mean of 802.89 $\pm$ 297.54 and the WD OSCC showed the least KI-67 LI of 368.67 $\pm$ 251.72. From the ANOVA table-2, there was significant difference between the three groups with respect to the mean Ki-67 LI ($P<0.05$). In order to find out among which pair of groups there exists a significant difference, in table 3 showed a significant difference between WD OSCC and PD OSCC ($P<0.05$). But, no significant difference existed between WD OSCC and MD OSCC ($P>0.05$) and between MD OSCC and PD OSCC ($P>0.05$)

Discussion:

The assessment of cell proliferation in many types of tumors is an important adjunct to histologically based tumor classification and has potential relevance as an indicator of treatment response and relapse.[4] Some investigators believe that the growth speed of a lesion indicates its future behavior pattern. Many studies have shown that Ki-67 monoclonal antibody staining is the best method for measuring cell proliferation. [8], [9]

Wangsa D et al (2008) [10] in their study have shown that Ki-67 expression level is also a potentially useful clinical marker for predicting recurrence in surgically treated stage I oral tongue SCC. Rafael Da Ros Motta et al (2009) [11] have proven that Ki-67 expression is significantly higher in oral epidermoid carcinoma patients with neck lymph node metastasis.

Table 1: Analysis of the Ki-67 LI in the three grades of OSCC

Group	Sample Size	Mean	Std Dev	95% Confidence Interval for Mean		Min	Max
				Lower Bound	Upper Bound		
WD OSCC	27	368.67	251.72	175.17	562.16	54.00	808.00
MD OSCC	27	802.89	297.54	574.18	1031.59	394.00	1502.00
PD OSCC	30	1243.40	810.77	663.41	1823.39	596.00	3442.00
Total	84	820.64	631.28	575.86	1065.43	54.00	3442.00

ANOVA, F=6.36, P=0.006, Significant (P<0.05)

Table 2: ANOVA

Mean Ki-67 LI	Sum of squares	df *	Mean square	F†	Significance
Between groups	**362865**	**2**	**1814307.570**	**6.360**	**0.006**
Within groups	**7131249**	**25**	**285249.972**		
Total	**10759864**	**27**			

***df: degree of freedom, † F: statistic**

Graph 1: Bar graph showing the comparison of mean Ki-67 LI in the three histological grades of OSCC.

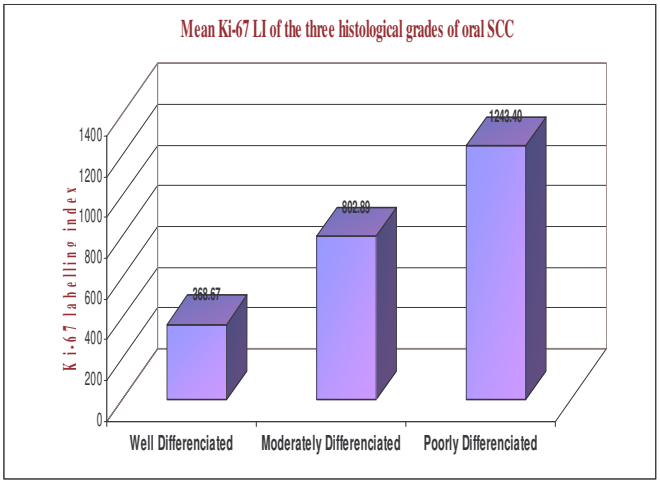

Figure 1: Ki-67 immunostaining of WD OSCC (400X)

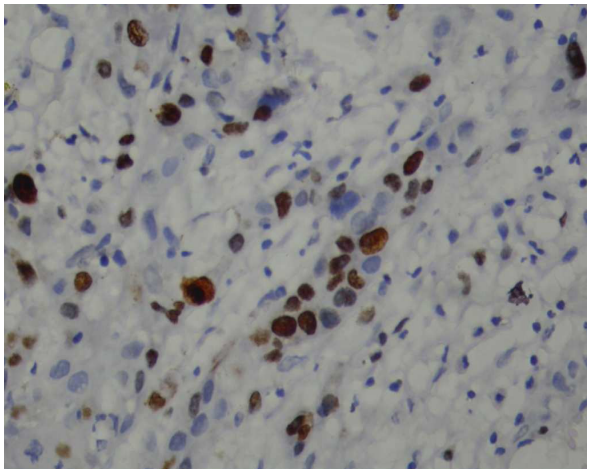

Figure 2: Ki-67 immunostaining of MD OSCC (400X)

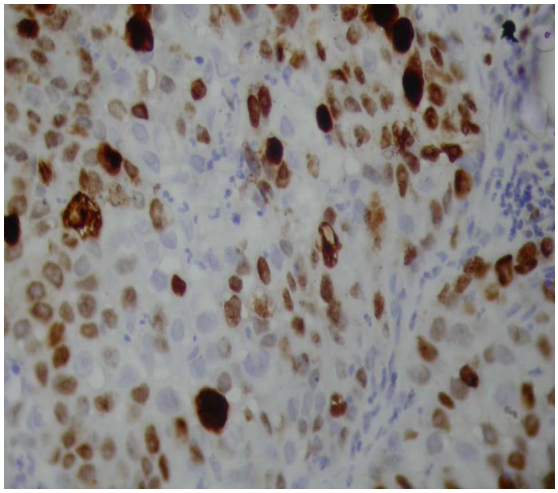

Figure 3: Ki-67 immunostaining of PD SCC (400X)

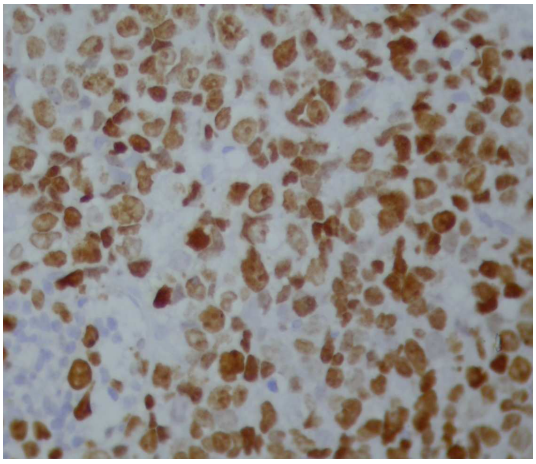

Table 3: Multiple comparisons test (Post-Hoc test) using Bonferroni method

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
WD OSCC	MD OSCC	-434.222	251.771	0.291	-1080.262	211.818
	PD OSCC	-874.733	245.396	0.005*	-1504.415	-245.051
MD OSCC	WD OSCC	434.222	251.771	0.291	-211.818	1080.262
	PD OSCC	-440.511	245.396	0.254	-1070.193	189.171
PD OSCC	WD OSCC	874.733	245.396	0.005*	245.051	1504.415
	MD OSCC	440.511	245.396	0.254	-189.171	1070.193

* denotes a significant difference

The monoclonal antibody Ki-67 was first described in 1983 by Johannes Gerdes and colleagues. The Ki-67 antigen was named after its place of characterization at Kiel in Germany and because the clone-producing antibody was grown in the 67th well of the tissue – culture plate. The Ki-67 protein, detected by immunolocalization of the Ki-67 antigen, is located in the nucleus and its gene is located on chromosome 10q25-ter. [1]

Li et al (1994) [4] have shown that although PCNA expression is related to the synthesis phase of the cell cycle, it does not necessarily have a close relationship with the cell cycle as it is also observed in the DNA repair process. Thus Ki-67 is a specific marker for cell proliferation; it is abundantly expressed in the S phase of cell cycle and disappears immediately after mitosis due to its short half-life. This makes Ki-67 antigen a preferable and trustworthy criterion in immunohistochemistry studies of lesions showing cell proliferation. [8], [9] Information on the growth fraction of tumors may be used in the assessment of tumor grade, and in all tumors which have been studied by Ki-67 staining, a highly significant correlation between Ki-67 staining and the degree of malignancy has been reported. Furthermore, a marked variation in the amount of Ki-67 within different tumor grades is seen, indicating that Ki-67 staining is useful in individual tumor diagnosis and prognosis. [5]

Six of our cases of WD OSCC did not stain despite the control being positive on repeated occasions. This finding was supported by Li et al [4] where they encountered inability in obtaining Ki-67 positive staining with four specimens (two unicystic and two follicular ameloblastomas). They speculated error in tissue processing. Varying results were obtained using Ki-67 marker to analyze the cellular kinetics of human OSCC when

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the malignancy grades obtained by routine histopathology and the proliferative indices obtained by Ki-67 immunostaining were compared.

Tumuluri V et al (2002) [1], Tumuluri V et al (2004) [12] and Kurokawa H et al (2005) [13] showed that the Ki-67 proliferative indices obtained correlated with the histological grading of OSCC. These results were in contrast with studies by Roland et al (1994)[14], Piffko J et al (1996) [15] and Bettendorf O et al (2002) [16]

Bryne M (1998) [17] advocated that the invasive tumor front is the most important area for prognostic determination of oral cancer. It consists of many molecular and morphological characteristics that reflect tumor progression better than other parts of the tumor. Several molecular events of importance for tumor spread such as gains and losses of adhesion molecules, secretion of proteolytic enzymes, increased cell proliferation and initiation of angiogenesis occur at the invasive front.

Tumuluri V et al [1] and Kurokawa H et al [13] have studied the relationship of Ki-67 LI at the invasive front of the OSCC with the histological grading and have concluded that expression of Ki-67 at the deep invasive tumor front of OSCC is associated with histologic grade of malignancy. Dissanayake U et al [18] and Piffkó J et al [15] have compared the Ki-67 LI at the invasive front and at the center of the tumor and have proved that Ki-67 LI at the invasive front is superior for prognostic purposes when compared to Ki-67 LI obtained from the center of the tumor.

In our study, quantification of the Ki-67 positive cells was performed in randomly selected fields, rather than the invasive tumor front or the center of the tumor sections. This approach was decided so as to facilitate the study of proliferative cells in incision biopsy specimens where obtaining the invasive tumor front is generally not possible; due to inability in locating the invasive tumor fronts in PD OSCC and also in extensive tumors.

Our results showed a generally increasing trend in the mean Ki-67 LI with increasing Broder's grade. However, a statistically significant difference was obtained only between Ki-67 LI of well and poorly differentiated OSCC. Ki-67 LI of moderately differentiated OSCC did not have statistically significant differences with either well or poorly differentiated OSCC. This observation reflects the subjective nature of the Broder's grading system.

Conclusion:

Our study shows that the tumor cell proliferation as measured by Ki-67 LI at randomly selected fields has a positive association with histologic grading in human oral squamous cell carcinoma. This finding ascertains that Ki-67 antigen can be used to determine the tumor behavior and prognosis in incisional biopsy material. Further studies considering a greater sample size and with other variants of human OSCC should be done to explore differences in their biological behavior and studies correlating the clinical course of the different histological grades of OSCC with their mean Ki-67 LI would be useful as prognostic indicators.

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