

ASSESSMENT OF PROLIFERATIVE POTENTIAL OF TUMOR CELLS USING KI-67 EXPRESSION AND MORPHOMETRICAL ANALYSIS FOR PROGNOSTICATION OF ORAL SQUAMOUS CELL CARCINOMAS

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ABSTRACT

Background: Cancer cells exhibit a characteristic intrinsic ability of uncontrolled cell proliferation which plays a vital role in tumor development and progression. The Ki-67 antigen expression is strictly associated with cell proliferation which detects the cells entering the cell cycle. In addition, computer aided image analysis provides an objective and highly reproducible histological evaluation of OSCC. Assessment of Ki-67 expression along with morphometry may help in early and precise diagnosis and prognostication of OSCC. **Aims:** To correlate the ki-67 expression and the morphometrical parameters of oral squamous cell carcinomas and to assess the efficacy of the correlation in OSCC prognostication. **Methods and material:** OSCC cases (n=105) were examined immuno histochemically using Ki-67 antigen The nuclear area (NA), cell area (CA), nuclear perimeter (NP) and cell perimeter (CP) assessed using "image J 1.34 software". The data was statistically analyzed. **Results:** Highly significant correlation was found between Ki-67 expression and the advancing grades of OSCC (p<.0001). NA (p=0.025), CA (p<.0001), NP (p=.027) and CP (p<.0001) of poorly differentiated OSCC were significantly lower than well differentiated and moderately differentiated OSCC. Follow up analysis revealed nuclear area and cell area to be higher in the recurrent cases than the non recurrent ones. **Conclusion:** Assessment of Ki-67 expression and morphometry help in early and precise diagnosis and prognostication of OSCC. A correlation between Ki-67 expression and morphometrical analysis could not be ascertained in this study. Further studies with a larger sample number may provide more definitive results.

INTRODUCTION

Oral cancer ranks eighth in the cancer incidence ranking worldwide. The World Health Organization anticipates a worldwide escalation in the incidence of oral squamous cell carcinomas (OSCC) in the subsequent decades [1]. Despite the steady improvements in treatment modalities, the 5 year survival rate of OSCC is about 55% and it continues to stand poor. This implies the need for early and accurate detection of OSCC in order to bring down the associated high morbidity and mortality rates [2]. Upon its onset, OSCC progresses imperceptibly, becoming evident only in a dangerously advanced state. This may be due to patients' negligence as it initially presents with no remarkable symptoms, or due to inappropriate diagnostic and prognostic assessment on the behalf of the clinician and the pathologist. The latter may be a result of lack of availability or utilization of reliable techniques for the same. The current scenario urges an enhanced comprehension of the tumor behavior.

The intrinsic capacities of limitless replicative potential and self-renewal are characteristics of cancer cells. Uncontrolled proliferation indicates that the cells are capable of acquiring further cellular alterations that contribute to full malignant phenotype. The expression of

the human Ki-67 protein is strictly associated with cell proliferation and is present during all active phases of cell cycle (G1, S, G2, and mitosis), but is absent from resting cells (G0). Thus it represents the growth fraction of the tumor and serves as a reliable nuclear marker for cell proliferation in malignancies [3]. Cell proliferation serves as a guide for prognostication of malignancies as a high proliferative activity is associated with a poor prognosis [2].

Histological examination is regarded as the gold standard for identifying and diagnosing OSCC [4]. However, there is wide variation among pathologists regarding the subjective evaluation of histopathological features. This necessitates the use of a more sophisticated technique of computer-assisted morphometry to investigate the cellular and nuclear changes in correlation with the clinical behavior of the lesions. There are many variables observable in microscopic images which are acquiescent to morphometrical analysis and the outcome is more objective, reliable and reproducible [5]. The morphometrical criteria considered in this study are: nuclear area, cell area, nuclear perimeter and cell perimeter.

Fairly extensive research has been conducted on the application of Ki-67 in head and neck cancer establishing its use as a proliferative marker^[2,3,6,7]. Morphometry has also been employed in a few studies in relation to the head and neck cancer^[4,5]. However, they have not been studied in conjunction before, for their implication in diagnosis and prognosis of head and neck squamous cell carcinoma. In this study, we attempt to determine the association between the Ki-67 expression and the morphometrical values as a useful adjunct to the routine histopathological examination. Our attempts are also directed to find out whether this association can enable us to use morphometrical values alone as an accurate and cost effective diagnostic tool for the underprivileged Indian oral cancer patients. To the best of our knowledge, such an association has not yet been established in the literature in relation to the head and neck squamous cell carcinoma.

MATERIAL AND METHODS

A retrospective cohort study

Ethics: Institutional ethics committee approval was obtained to carry out the study (IEC 526/2012)

Inclusion criteria: Only the cases which were histologically confirmed cases of OSCC of the bucco-alveolar complex and the floor of the mouth and the patents in whom the treatment (radiotherapy or chemotherapy) had not begun at the time of initial diagnosis were included.

Exclusion criteria: Cases of oral cancer other than oral squamous cell carcinoma, cases of oral squamous cell carcinoma undergoing treatment, cases with history of recurrence of oral squamous carcinoma and cases of oral squamous cell carcinoma with systemic conditions were excluded.

Methodology

A total of 105 formalin fixed paraffin embedded (FFPE) cases of OSCC were studied. 35 cases each of well, moderate and poorly differentiated OSCC of bucco-alveolar mucosal complex and floor of the mouth were studied. The selected cases were diagnosed and treated at the University Hospital from the year 2008 to 2013. 5 tonsil tissue specimens were used as controls along with 5 cases of healthy oral mucosal tissues. Relevant clinicopathologic details including the tumor staging, the histologic grading, and the development of recurrence or metastasis were attained from the medical record files.

Immunohistochemistry

Thin sections of 4µm were cut from FFPE tumor blocks. The section were mounted on amino-propyl-tri-ethoxy-silane (APES) coated glass slides and stained with monoclonal antibody against ki67 (RTU - Ki67 – MM1: Novacastra) using indirect streptavidin biotin immunoperoxidase technique. Tissue sections obtained from tonsil tissue specimens were taken as positive controls for Ki-67 immunohistochemistry. As a negative control for immunohistochemical procedures the primary antibody was replaced with normal mouse IgG in appropriate concentration^[2].

Evaluation of Ki -67 expression: The immunoreactivity of Ki-67 was nuclear. The nuclei with clear brown color,

regardless of staining intensity, were regarded as positive. The positive cells of the tumor cells were evaluated in 5 representative fields at 40x magnification. The histological sections with uniform and good intensity staining were assessed. (Figure 1 a,b) The histological section of tonsil tissue stained with Ki-67 was used as positive control and confirmed against a negative control (Figure 2a,b). The ki-67 labelling index was calculated using the formula^[8]: (Ki67-positive) / (Ki67-positive + Ki67-negative) x 100

Based on the labeling index, the sections were scored from 1 to 3 for ki-67 expression as follows^[9]:

Ki-67 expression	Extent of proliferation	Percentage of positive cells
1	High	>50 %
2	Moderate	30 to 50%
3	Low	<30%

Morphometry: Tissue sections of 5µm thickness were cut from FFPE tissue blocks, stained with Harris's Haematoxylin and Eosin and subsequently subjected to morphometric analysis. Only clearly defined cells were measured. The 4 morphometrical parameters considered were: Nuclear area (NA), cell area (CA), nuclear perimeter (NP) and cell perimeter (CP)

The scale for morphometrical analysis was standardized using an eye piece graticule and a stage grid in 40X magnification. For each case, pictures of 3 fields were taken under 40X magnification. Ten clearly defined cells were analyzed from each field. A total of 3150 cells were morphometrically analyzed. Image analysis was done using – "Image J 1.34 software"^[10] available at website: <http://rsb.info.nih.gov/ji/>.

In order to assess the slides in image J, the images were captured onto the hard drive of the computer, following which they can be opened in Image J for evaluation, using the various tools provided in the panel^[8,11].

Statistical analysis: SPSS (Statistical Package for Social Sciences) version 16.0 for windows was used. *P*-value (*p*) <0.05 was considered significant for all statistical analysis.

- Inter-class correlation (ICC) carried out for inter-observer reproducibility between two observers.
- Chi square test was done to study the association between ki67 labelling index and Ki-67 expression.
- One- way ANOVA (Analysis of Variance) was used for comparing the mean Ki-67 labeling index among the different grades. Comparison of the mean ki-67 labeling index between groups was made using multiple comparison test by Tukey-HSD procedure
- One- way ANOVA (Analysis of Variance) was used for comparing the morphometrical parameters for multiple groups. Comparison of the mean nuclear and cellular area and diameter values between groups was made using multiple comparison test by Tukey-HSD procedure.
- Pearson Correlation test was done to analyse the association between ki-67 expression and each of the 4 morphometrical parameters.
- Chi-square test was applied to study the correlation between Ki-67 expression and OSCC grades.

- Chi-square analysis was done subsequently to correlate the Ki-67 expression with the disease recurrence.

RESULTS

In the test group which included 105 OSCC cases, positive Ki-67 expression in the nuclei of proliferating tumor epithelial cells was found positive in all the cases (100%). 5 tonsil cases were used as controls. 5 cases of normal oral mucosa were also used for comparative assessment of staining. The inter observer reproducibility was analysed with inter-class correlation which revealed a good reproducibility between the 2 observers (ICC=.925)

Among the 105 cases of squamous cell carcinomas in 49/105 (47%) 30-50% of tumor cell expressed Ki 67. Among the 35 cases of well differentiated tumors in 13/35 (37%) 30-50% of tumor cells expressed Ki 67 while in 17/35 (49%) cases <30% of cells were positive for the biomarker. Among the 35 cases of moderately differentiated tumors in 20/35 (57%) 30-50% of tumor cells expressed Ki 67 while in 1/35 (2%) cases <30% of cells were positive for the biomarker. Among the 35 cases of poorly differentiated tumors in 16/35 (46%) 30-50% of tumor cells expressed Ki 67 while in 16/35 (46%) cases <30% of cells were positive for the biomarker. On statistical analysis with chi-square test, a highly significant correlation was found between Ki-67 expression and the advancing grades of OSCC. (p<.001)(Table 1)

The Ki-67 expression and labelling index was assessed in the 105 cases of oral squamous cell carcinomas. The mean labeling index among the well, moderate and poorly differentiated OSCC was 29.84 ± 13.04 ; 48.10 ± 13.41 and 32.015 ± 13.89 respectively. A one way Anova test showed that there was a significant (p <.001) variation in mean Ki-67 labeling index between the tumor grades. A post –hoc test with Tukeys HSD showed that there was a significant (p <.001) association between Ki-67 labelling index of well differentiated and moderately differentiated and that between moderately differentiated and poorly differentiated tumors.(Table 2 and Table 3)

Ki-67 expression and Ki-67 labelling index also showed a highly significant association through Chi-square test (p<.001).

In addition, an analysis of the ki-67 expression and the intra-oral site of OSCC was also done. Ki-67 expression of nine different sites of the oral cavity was assessed and its mode was calculated. The tongue (dorsal/ventral aspect), vestibule and floor of the mouth and buccal mucosa with alveolus show lowest ki-67 expression^[1], whereas angle of mandible, alveolar region and buccal mucosa show moderate ki-67 expression^[2] and tongue (lateral border), alveolar region and lips show highest ki-67 expression^[3] and least degree of tumoral differentiation. (Table 4)

Nuclear area: The mean nuclear area of poorly differentiated OSCC was found to be $162.04\mu^2$. One Way ANOVA test showed the value of mean nuclear area to be statistically significant among the grades of OSCC (p= 0.025). Comparison between the groups using Post-Hoc test with Tuckey HSD method showed the value of poorly differentiated OSCC to be significantly lower than well differentiated OSCC (p= 0.05) and moderately differentiated OSCC (p= 0.042)

Similarly, the mean cell area (p<.0001), mean nuclear perimeter (p= 0.27) and mean cell perimeter (p<.0001) were found to be statistically significant among the grades of OSCC. Comparison between the groups using Post-Hoc test with Tuckey HSD method has been depicted in Table 5 and 6

However no statistically significant correlation was seen between ki-67 and any of the 4 morphometrical parameters. (Table 7)

Follow up and recurrence analysis:

Among the 105 cases, 62 were available for follow up, out of which 8 patients showed loco-regional recurrence (12.9%). After their morphometrical analysis it was seen that nuclear area, cell area and nuclear perimeter were higher in the recurrent cases as compared to the non recurrent ones. (Figures 3 and 4)

Recurrence was also correlated with Ki-67 expression with chi-square analysis, which indicated it to be statistically insignificant.

Table 1: Association between tumor grade and Ki-67 expression

Tumor Grade	High Proliferation	Moderate Proliferation	Low Proliferation	Total	X2 Value	df	P value
Well	5 (14%)	13 (37%)	17 (49%)	35	25.050 ^a	4	.000
Moderate	14 (40%)	20 (57%)	1(3%)	35			
Poor	3 (8%)	16 (46%)	16 (46%)	35			

Table 2: One way Anova to compare the mean Ki-67 labelling index

	Sum of squares	df	Mean square	F	Sig
Between groups	.6964	2	3482.066	19.224	.000
Within groups	18475.855	102	181.136		
total	25439.988	104			

Table 3: Comparison of the mean labelling index among the different grades Tukey HSD

(I) Grade	(J) Grade	Mean difference (I-J)	Std. Error	Sig.	95% confidence interval	
					Lower bound	Upper bound
Well	Moderate	-18.25729*	3.21724	.000	-25.9092	-10.6054
	Poor	-2.16686*	3.21724	.779	-9.8188	5.4850
Moderate	Well	18.25729	3.21724	.000	10.6054	25.9092
	Poor	16.09043*	3.21724	.000	8.4385	23.7423
Poor	well	2.16686	3.21724	.779	-5.4850	9.8188
	Moderate	-16.09043*	3.21724	.000	-23.7423	-8.4385

* The mean difference is significant at the .05 level.

Table 4: showing the relationship of Ki-67 expression with site of OSCC cases

site	Number of cases of Well differentiated OSCC	Number of cases of Moderately differentiated OSCC	Number of cases of Poorly differentiated OSCC	Ki-67 Expression occurring with highest frequency (Mode)
Tongue - lateral border (left/right)	2	14	6	3
Tongue - ventral/dorsal aspect	2	1		1
Buccal mucosa	9	6	4	2
Buccal mucosa and alveolus	4	1		1,2
Alveolar region	5	6	2	3
Retromolar region	2	1		2
Angle of mandible	1	1		2
lips	1		3	3
Vestibule and floor of the mouth		1	3	1
Total cases (With the details of the site of OSCC)	26/35	31/35	18/35	

(Ki-67 expression: 1 = high proliferation, 2 = moderate proliferation, 3 = low proliferation)

Table 5: Statistical analysis using One – Way ANOVA test and multiple comparisons test (Post-Hoc test) with Tukey HSD method (values in micron²)

Criteria	Grade of OSCC	Mean	Standard deviation	Confidence limit	P-value
Nuclear Area	Well differentiated (a)	190.77	54.48	172.05, 209.49	.025 Post hoc: (a) and (c)= .05 (b) and (c)= .042
	Moderately differentiated (b)	192.11	46.86	176.02, 208.21	
	Poorly differentiated (c)	162.04	52.57	1443.98, 180.10	
Cell Area	Well differentiated (a)	542.29	139.91	494.23, 590.35	<.0001 Post hoc: (a)and (c)= <.0001 (b)and (c)= <.0001
	Moderately differentiated (b)	553.98	127.48	510.19, 597.77	
	Poorly differentiated (c)	414.72	127.68	370.85, 458.58	

Table 6: Statistical analysis using One – Way ANOVA test and multiple comparisons test (Post-Hoc test) with Tukey HSD method (values in micron²)

Criteria	Grade of OSCC	Mean	Standard deviation	Confidence limit	P-value
Nuclear Perimeter	Well differentiated (a)	48.28	6.47	46.06, 50.51	.027 Post hoc: (b) and (c)= .03
	Moderately differentiated (b)	48.92	5.86	46.91, 50.94	
	Poorly differentiated (c)	44.88	7.49	42.30, 47.46	
Cell Perimeter	Well differentiated (a)	86.82	12.17	82.63, 91.00	<.0001 Post hoc: (a) and (c)= .001 (b) and (c) <.0001
	Moderately differentiated (b)	88.88	9.95	85.46, 92.30	
	Poorly differentiated (c)	76.38	13.08	71.89, 80.88	

Table 7: Showing statistically insignificant correlation between Ki-67 labelling index and the morphometrical parameters

Morphometrical Parameter	R (Pearson correlation Coefficient)
Nuclear area (NA)	.123
Cell area (CA)	.194
Nuclear perimeter	.148
Cell perimeter	.207

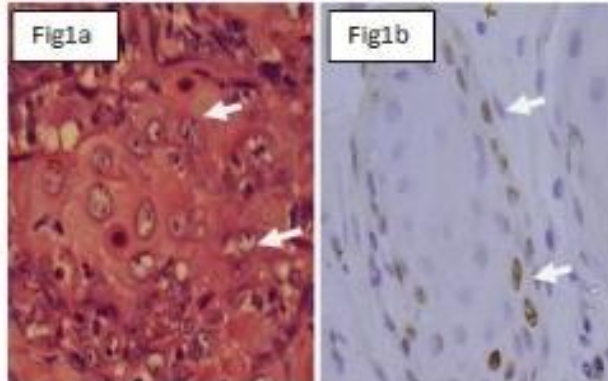


Fig 1a: Photomicrograph of well differentiated Squamous Cell Carcinoma (H & E staining, 40x). Arrows indicate the tumor epithelial cells.

Fig1b: Photomicrograph of well differentiated Squamous Cell Carcinoma (IHC staining with Ki-67, 40x). The arrows indicate Ki-67 staining taken up by the cells undergoing proliferation.

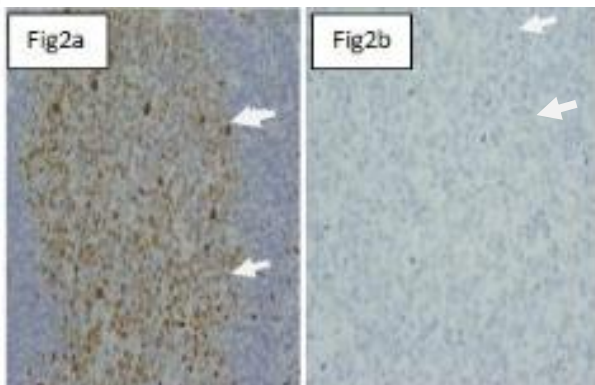


Fig 2a: Photomicrograph of tonsil tissue used as positive control stained with Ki-67 (IHC staining with ki-67, 20x). The arrows indicate Ki-67 staining taken up by the tonsillar cells undergoing proliferation.

Fig 2b: Photomicrograph of tonsil tissue stained immunohistochemically with the exclusion of primary antibody, used as negative control (20x)

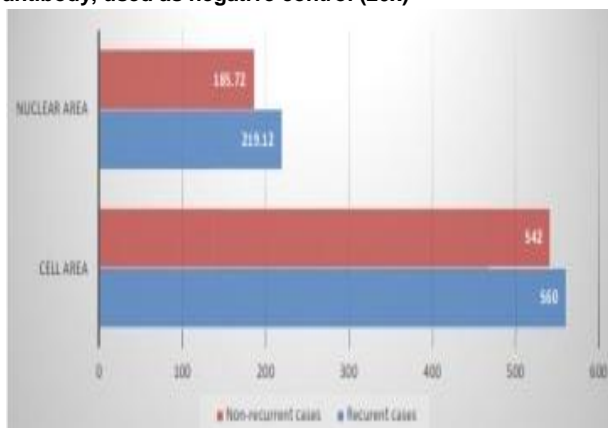


Fig 3: Showing comparison between recurrent and non recurrent cases with respect to nuclear area (NA) and cell area (CA) (values in micron²)

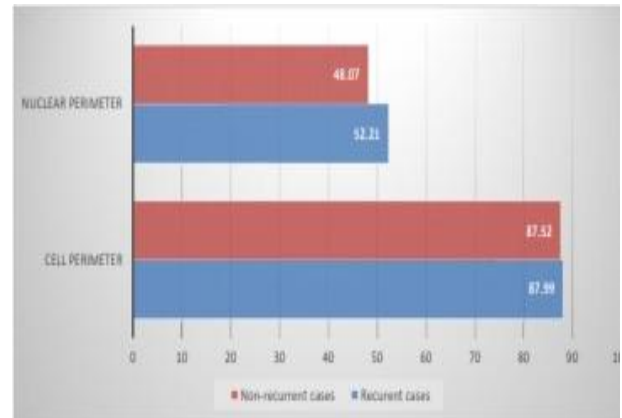


Fig4: Showing comparison between recurrent and non recurrent cases with respect to nuclear perimeter (NP) and cell perimeter (CP) (values in micron²)

DISCUSSION

Oral squamous cell carcinoma (OSCC) is known for its unpredictable progression and severe damage of the tissues involved. Conventionally, OSCC is evaluated with clinical staging and histological grading system, which are essentially subjective and not efficiently reproducible. It is certain that it will take more than just routine histological methods to detect and control this disease before its characteristic progression to serious impairment.

Expression of proliferation markers such as ki-67 for lesions of oral mucosa has been shown to be correlated with the severity of the lesion^[12]. The expression of the human Ki-67 protein, a nuclear marker, provides an accurate estimation of the tumour growth rate in a relatively cost and time effective manner^[7].

A study conducted by Dragomir L.P et al. on 34 cases of OSCC, showed that the increased expression of Ki-67 was associated with the decrease of the degree of tumoral differentiation and with high degree dysplasia^[13]. Recently, the findings of Dwivedi N et al^[14] confirmed that the expression of Ki-67 provides an objective criteria for determining the histological grading of OSCC. They also assessed the severity of epithelial dysplasia using Ki-67 expression.

In our study the immuno expression of Ki-67 was seen in all the 105 cases (100%) of OSCC and our results were closely similar to the observations of Kannan et al.^[12], Premlatha et al.^[15] and Bryant et al.^[16] On statistical analysis with chi-square test, a highly significant correlation was found between ki-67 expression and the advancing grades of OSCC ($p < .001$). This indicated that the rate of cell proliferation tends to increase with the decrease in the degree of tumor differentiation in OSCC. We correlated the Ki-67 expression with the site of OSCC to attain the site with highest ki-67 expression. Nine different sites of the oral cavity were assessed namely Tongue - lateral border (left/right), Tongue - ventral/dorsal aspect, Buccal mucosa, Buccal mucosa with alveolus, Alveolar region, Retro molar region, Angle of mandible, lips, Vestibule and floor of the mouth. Mode was calculated for the ki-67 expression among the different sites. Results showed that tongue (lateral

border), alveolar region and lips show highest ki-67 expression and least degree of tumoral differentiation. Our study also showed a highly significant correlation between ki-67 labelling index and ki-67 expression ($p < .001$). Furthermore, the Ki-67 labeling index was found to increase with the advancing grades of OSCC. The moderately differentiated OSCC showed a significantly higher proliferation than well differentiated OSCC ($p < .001$). Thus higher Ki-67 labelling index could indicate a poor prognosis, as was demonstrated by Maheshwari et al.^[9] Our results supported the cogency of Ki-67 as a potential proliferative marker for OSCC. The analysis was quick and easy and there was good reproducibility between the two different observers. (ICC=.925)

In addition, we studied the expression of Ki-67 in 5 specimen of normal oral mucosa and the expression was found to be present in the basal and parabasal layers. This finding was similar to that made by Rendon et al.^[17] and Birajdar et al.^[18]

Although certain other immunohistochemical markers such as PCNA, Cyclin D and CENP-F may also be used to assess cellular proliferation, the present study uses Ki-67 due to its intense nuclear staining which is evident in all the phases of the cell cycle except G0 phase and the background staining is nominal^[3,7,9]. It can thus be interpreted efficiently with ease.

The present study further attempts to correlate ki-67 expression with computer-aided morphometrical analysis, in order to develop an index which may enable the use morphometrical values alone as an accurate and cost effective diagnostic tool for the underprivileged Indian oral cancer patients. Although several systems are available for morphometrical image analysis. Image J serves as a more cost effective alternative, developed at the National Institutes of Health (NIH)^[19,21]. The validity of Image J has been demonstrated in our previous study conducted with the same set of cases^[22]. The previous study was carried out with 6 morphometrical parameters, out of which the current study has considered 4 parameters for further analysis, namely nuclear area, cell area, nuclear perimeter and a cell perimeter. These parameters have been particularly chosen for the current study as they can be calculated with ease and minimum time consumption using the Image j software. The reliability of these criteria has been emphasized in previous literature, however this is the first study to take account of all the four criteria for analysis.

Giardina et al.^[23] conducted a study to highlight the significance of nuclear morphometry in OSCC. They analysed 30 cases to study the relationship between nuclear shape and survival. They established that morphometrical analysis could successfully distinguish patients of the two groups of short term and long term survival with only a 10% error.

Similarly, DB Nandini and RV Subramanyam^[24] conducted a study using computer-assisted microscopy on nuclear features in oral squamous cell carcinoma and emphasized the reliability of computer-assisted nuclear morphometry in OSCC grading.

These studies coordinate with the morphometrical results depicted in our previous study^[22]. NA for poorly

differentiated OSCC, $162.04 \mu^2$, was significantly lower than both well and moderately differentiated OSCC. Similarly CA of poorly differentiated OSCC was $414.72 \mu^2$ which was significantly lower than both well and moderately differentiated OSCC. NP for poorly differentiated OSCC was $44.88 \mu^2$ which was significantly lower than moderately differentiated OSCC. CP of poorly differentiated OSCC was significantly lower than well differentiated OSCC as well as moderately differentiated OSCC.

These morphometrical values can provide more reliable information to the clinician and are more comprehensive for the patients. However no statistically significant association was observed between ki-67 and any of the 4 morphometrical parameters of the study. This might have been due to less number of cases or a consequence of insufficient patient follow up details. Further research overcoming the limitations of the study could be more effectual.

Taken together, the data from our study adds weight to the growing body of evidence that Ki-67 is a powerful tool to interpret the proliferative potential of the advancing grades of OSCC and that 4 simple yet reliable morphometrical parameters can help in prognostication of OSCC.

CONCLUSION

The study showed Ki-67 to be a reliable proliferative marker for OSCC, along with a strong diagnostic and prognostic significance of four morphometrical parameters in the advancing grades of OSCC. However the association of Ki-67 expression with the computer aided image analysis could not be ascertained. Further studies based on correlating computer aided image analysis and immunohistochemistry may help to develop a simple yet promising adjunct to routine histological examination which helps in diagnosing and understanding OSCC and can be highly valuable tool in predicting an accurate and timely prognosis in order to formulate an effective treatment plan according to the individual treatment needs of the patient.

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Conflict of Interest: Nil

REFERENCES

1. World Health Organization. The World Oral Health Report. Geneva: World Health Organization; 2003; 6-7.
2. Pich A, Chiusa L & Navone R. Prognostic relevance of cell proliferation in head and neck tumors. *Ann Oncol* 2004; 15: 1319–29.
3. Boas D.S, Takiya C.M, Sampaio T.L.C, Ribeiro L.C, Ramos E.A.G, Cabra M.G et al. Immunohistochemical detection of Ki-67 is not associated with tumor-infiltrating macrophages and cyclooxygenase-2 in oral squamous cell carcinoma; *J Oral Pathol Med* 2010; 39: 565–70.

4. Acha A, Ruesga MT, Rodríguez MJ, de Pancorbo MA M, Aguirre JM. Applications of the oral scraped (exfoliative) cytology in oral cancer and precancer. *Med Oral Patol Oral Cir Bucal* 2005; 10: 95–102
5. T Smitha, P Sharada, and HC Girish. Morphometry of the basal cell layer of oral leukoplakia and oral squamous cell carcinoma using computer-aided image analysis. *J Oral MaxillofacPathol* 2011; 15(1): 26–33.
6. Daniel F.I, Fava M, Hoffmann R, Campos M, Yurgel L. Main Molecular Markers of Oral Squamous Cell Carcinoma. *Applied Cancer Research* 2010; 30(3): 279-88.
7. Myoung H, Kim M, Lee J.H, Ok Y.J, Paeng J.Y, Yun P.Y. Correlation of proliferative markers (Ki-67 and PCNA) with survival and lymph node metastasis in oral squamous cell carcinoma: a clinical and histopathological analysis of 113 patients. *Int. J. Oral Maxillofac. Surg* 2006; 35: 1005–10.
8. Mukherjee S, Katarkar A, Ray JG, Chaudhuri K. 2012. Immunohistochemical markers to differentiate oral precancerous and cancerous lesion: an integrated tissue based microscopic analysis. In: Méndez-Vilas A, editor. Current microscopy contributions to advances in science and technology: Formatex Research Center, 433-38.
9. Maheshwari, Veena, et al. "Prognostic and Predictive Impact of Ki-67 in Premalignant and Malignant Squamous Cell Lesions of Oral Cavity." *Int. J. Head Neck Surg* 4 2013: 61-65.
10. Natarajan S, Mahajan S, Boaz K, George T. Prediction of lymph node metastases by preoperative nuclear morphometry in oral squamous cell carcinoma: a comparative image analysis study. *Indian J Cancer* 2010 Oct-Dec; 47(4): 406-11.
11. Khandelwal S, Solomon MC. Cytomorphological analysis of keratinocytes in oral smears from tobacco users and oral squamous cell carcinoma lesions - A histochemical approach. *Int J Oral Sci* 2010; 2(1): 45-52.
12. Kannan S, Jagadeesh G Chandran, Pillai K R, Mathew B, Sujathan K, Nalinakumary K. R, Nair M.K. Expression of p53 in leukoplakia and squamous cell carcinoma of the oral mucosa: correlation with expression of Ki67. *J ClinPathol: MolPathol* 1996; 49: 170-75.
13. Dragomir LP, Simionescu C, MĂfrcĂfritescu C, Stepan A, Dragomir IM, Popescu MR. P53, p16 and Ki67 immunoexpression in oral squamous carcinomas. *Rom J MorpholEmbryol* 2012; 53:89-93.
14. Dwivedi N, Chandra S, Kashyap B, Raj V, Agarwal A. Suprabasal expression of Ki-67 as a marker for the severity of oral epithelial dysplasia and oral squamous cell carcinoma. *ContempClin Dent* 2013; 4: 7-12.
15. Premalatha B.R., K. Uma. Analysis of KI-67 antigen in human Oral Squamous Cell Carcinoma – An immunohistochemical study. *J. Int Oral Health* 2010; 2(1): 9-16.
16. Bryant RJ, Banks PM, O'Malley DP. Ki67 staining pattern as a diagnostic tool in the evaluation of lymphoproliferative disorders. *Histopathology* 2006 Apr; 48(5): 505-15.
17. Torres-Rendon A, Roy S, Craig GT, Speight PM. Expression of Mcm2, geminin and Ki67 in normal oral mucosa, oral epithelial dysplasias and their corresponding squamous-cell carcinomas. *Br J Cancer* 2009 Apr 7; 100(7): 1128-34.
18. Birajdar SS, Radhika M B, Paremala K, Sudhakara M, Soumya M, Gadivan M. Expression of Ki-67 in normal oral epithelium, leukoplakic oral epithelium and oral squamous cell carcinoma. *J Oral MaxillofacPathol* 2014; 18: 169-76.
19. Noguchi M, Kikuchi H, Ishibashi M, Noda S. Percentage of the positive area of bone metastasis is an independent predictor of disease death in advanced prostate cancer. *Br J Cancer* 2003; 88: 195-201.
20. Ho CC, Yang XW, Lee TL, Liao PH, Yang SH, Tsai CH, et al. Activation of p53 signalling in acetylsalicylic acid-induced apoptosis in OC2 human oral cancer cells. *Eur J Clin Invest* 2003; 33: 875- 82.
21. Tchoukalova YD, Harteneck DA, Karwoski RA, Tarara J, Jensen MD. A quick, reliable, and automated method for fat cell sizing. *J Lipid Res* 2003; 44: 1795-01.
22. Rakheja M, Chauhan A, Guddattu V and Solomon M. Morphometrical characteristics of tumor cells are prognostic determinants for oral squamous cell carcinomas. *International Journal of Current Research* 2014; 6(11):9576-82.
23. Giardina C, Caniglia DM, D'Aprile M, Lettini T, Serio G, Cipriani T, Ricco R, PesceDelfino V. Nuclear morphometry in squamous cell carcinoma (SCC) of the tongue. *Eur J Cancer B Oral Oncol* 1996 Mar; 32B(2): 91-96.
24. Nandini DB, Subramanyam RV. Nuclear features in oral squamous cell carcinoma. *J Oral MaxillofacPathol* 2011 May; 15(2): 177-81.