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Original Research Article

A study of Ki-67 expression in oral squamous cell carcinoma

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ABSTRACT

Introduction: Oral squamous cell carcinoma (OSCC) is a common malignancy in the Indian subcontinent. Various epidemiological and histologic parameters aid in prediction of prognosis but are not always reliable. One of the most important mechanisms in oncogenesis is cell proliferation and Ki-67 is a useful marker for its measurement.
Aims and Objectives: To compare the expression of ki-67 with histologic grade, pT staging, lymph node status, perineural invasion (PNI) and lymphovascular invasion (LVI).
Materials and Methods: 50 cases of histologically confirmed oral squamous cell carcinoma were included in the study. ki-67 expression labeling index was measured and correlated with the above mentioned histologic parameters.
Results: There was an increase in the mean ki-67 expression with increasing histologic grade (p-value 0.047). No statistically significant correlation of ki-67 expression was seen with pathologic T stage (p-value 0.733), lymph node status (p-value 0.933), lymphovascular invasion (p-value 0.534) and perineural invasion (p-value 0.573).
Conclusion: Mean ki-67 expression was found to be statistically significant with histologic grade. No statistically significant correlation of ki-67 expression was seen with pathologic T staging, lymph node status, presence of lymphovascular invasion and perineural invasion. Further studies correlating ki-67 expression with PNI/LVI and other histologic parameters in OSCC may aid in early diagnosis and prediction of the same.

1. Introduction

Oral cancers are malignant neoplasms that affect the structures of the mouth. Of these oral cancers, more than 90% are squamous cell carcinomas arising in the mucous membranes of the mouth and oropharynx.[1]

It is an important global health concern accounting for an estimated 275,000 cases and 128,000 deaths annually. India has one of the highest incidences of oral cancer.[2] A recent survey of cancer mortality in India shows cancer of the oral cavity as the leading cause of mortality in men, and it is responsible for 22.9% of cancer-related deaths.[3]

GLOBOCAN 2012 data suggests an incidence of 77003 cases annually with crude rate of 6.1 per 100,000 and estimated 52067 deaths annually with crude rate of 4.1 per 100,000. 5-year survival rates are as high as 80% for patients treated early.[4] It is a multifactorial disease. It has a remarkable incidence worldwide and has fairly burdensome prognosis, encouraging further research on factors that may modify disease outcome.[5]

The unpredictable behavior of OSCCs has led clinicians to look for reliable parameters to be applied as predictors of prognosis that can be divided into epidemiologic parameters (age, sex, race, alcohol, and/ or tobacco intake), clinical parameters (T classification, TNM (tumor-node-metastasis) stage, site), and histologic parameters (lymphovascular invasion, perivascular invasion, grading, pattern of invasion,

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tumor thickness).[6] These conventional prognostic markers, though widely accepted are not always well used in the diagnosis and prediction of OSCC.[7] Patients with PNI (perineural invasion) and LVI (l ymphovascular invasion), have the worst prognosis.[8] Previous studies on oral cancer indicated that the prognostic factors and the failure patterns vary across different primary subsites.[9,10] Despite of these findings, no report is yet available on the importance of pathological features at different primary subsites.

Cell proliferation is considered one of the most important mechanisms in oncogenesis.[11] Ki-67 is present in all proliferating cells, but absent in resting cells, which indicated that Ki-67 might be a potential tool for quick estimation of the proportion of proliferating cells in a neoplasm.[12]

The rate of cell proliferation measured by Ki-67 may be useful in characterizing the histological grade in oral squamous cell carcinoma. Further studies are needed to evaluate the behavior of well-differentiated tumors with high rate of cell proliferation to define the best therapeutic approach in these patients. Not many studies have been done especially in India which focuses on the expression of Ki-67 in oral squamous cell carcinoma. Also, the expression of Ki-67 with grade of oral squamous cell carcinoma, pathological stage, presence or absence of LVI and presence or absence of PNI need to be studied for the knowledge and exact function of Ki-67. This information regarding kinetic data will aid in patient care.

In this study we wish to compare the expression of ki-67 and the various histopathological prognostic factors of oral squamous cell carcinoma.

2. Materials and Methods

The present study was conducted in Department of Pathology at SRMS Medical College and Hospital, Bareilly, a tertiary health care centre. 50 consecutive surgically resected specimen of histologically confirmed oral cavity squamous cell carcinoma were included in the study. These specimens were received in pathology lab from the Department of Otorhinolaryngology in 10% formalin. After adequate fixation for 12-24 hours, sections were taken and were then submitted for routine processing, followed by paraffin embedding. 5μm thick slices were cut from the sections submitted, to be stained with Haematoxylin and Eosin. AJCC pTN definitions for oral squamous cell carcinoma were used for the staging of the specimens.[13]

2.1. Histologic Grade

OSCC specimen was histologically graded as well differentiated, moderately differentiated and poorly differentiated Squamous Cell Carcinomas according to the differentiation of cells and the resemblance of neoplastic cells to that of epithelial cells.

2.2. Broder's (1920) classification:[14]

Accordingly, tumors were graded on the basis of degree of differentiation and keratinization of tumor cells into:-

2.3. Immunohistochemical staining method (Biogenex)

Sections of thickness 2-3microns were cut, floated to positively charged slides, incubated for 37°C for one day and further incubated at 58°C overnight, deparaffinised, and rinsed in distilled water. Antigen retrieval was done by heating in a microwave oven, cooled to room temperature and rinsed. Sections were washed in TBS (Tris-buffered saline pH-7.6), treated with peroxidase block to block non-specific reaction with the other tissue antigens. Excess power block was then drained, treated with primary antibody for Ki-67 to identify the tumor markers by antigen-antibody reaction, treated with SS(super sensitive) enhancer to enhance the reaction, treated with SS polymer (secondary antibody) to elongate chain and to label the enzyme. Then sections were treated with DAB (diaminobenzidine) working solution to give brown colour to the antigen, counter stained with Harris hematoxylin for 1 minute, washed, deparaffinised, cleared and mounted with DPX.

Ki-67 activity was quantified by selecting the most densely and evenly labeled areas in the sections and assessing the labeling index from the ratio of the number of cells stained by Ki-67 to the total number of cells counted per section [minimum of 1000 cells at high power ($\times$40). All nuclei with diffuse or dot-like brown nuclear staining irrespective of staining intensity was rated as positive for Ki-67.

$$Labelling\ index = \frac{No.\ of\ cells\ stained\ by\ ki{-}67}{Total\ no.\ of\ cells\ counted} \times 100$$

2.4. Statistical analysis

Data was analyzed using Statistical Package for Social Sciences (SPSS), version 23. Results for continuous and categorical variables are presented as mean $\pm$ standard deviation and as number (percentage). We also calculated the correlations among Ki-67 expressions in tumor. The level $P < 0.05$ was considered as the cutoff value or significance.

3. Results and Observations

Out of the 50 cases of OSCC studied, maximum belonged to the age group 41-50yrs (30%), with minimum cases seen in ages 61-70yrs and more than 70 yrs (8%each). Mean age observed was 48.4 yrs.

Among the 50 cases studied, OSCC was seen to be more prevalent in males (72%) with only 14 cases (28%) seen in females. Buccalmucosa (46%) and tongue (44%) were observed to be the most common sites in the oral cavity. 32 cases (64%) were well differentiated, 13 cases (26%) were moderately differentiated and only 5 cases (10%) were

Table 1:

Well differentiated SCC	Relatively mature tumour cells with few nuclear aberrations and with the presence of keratin pearls and/or individual cell keratinization.
Moderately differentiated SCC	The presence of tumour cells exhibiting a wide range of differentiation. Keratinization was occasionally present and nuclear aberrations were moderately abundant.
Poorly differentiated SCC	Disorderly and poorly differentiated tumour cells with no tendency to keratinization. Nuclear aberrations were abundant.

poorly differentiated.

Among the 50 cases of oral squamous cell carcinoma studied, 21 tumors (42%) were 2-4cm in greatest dimension(pT2) while only 5 (10%) were seen to be invading through cortical bone, into deep/extrinsic muscle of tongue, maxillary sinus, or skin of face (pT4). 30 cases (60%) showed no regional lymph node metastasis (N0), 26% cases showed metastasis in a single ipsilateral lymph node, 3 cm or less in greatest dimension (N1), and 14% cases showed metastasis in ipsilateral/contralateral lymph nodes measuring 3-6cm in greatest dimension.

LVI was absent in 28 cases (56%) and present in 22 cases (44%). PNI was absent in 36 cases (72%) and present in 14 cases (28%). LVI was seen in 4/5 cases (80%) of poorly differentiated and 7/32 cases (22%) of well differentiated OSCC with P-value=0.003. PNI was seen in 3/5 cases (60%) of poorly differentiated and 3/32 cases (9%) of well differentiated OSCC with P value=0.001 calculated by using modified chi-square test (Figure 1).

The mean of ki-67 expression in well differentiated was 44.2, 56.5 in moderately differentiated and 69.8 in poorly differentiated OSCC. P value = 0.047 was calculated by Paired-T test**.** The mean of ki-67 expression was maximum in T1 stage (56.2) and minimum in T4 stage (43.9) with p-value 0.733. The mean of ki-67 expression was maximum in N1 stage (55.7) and minimum in N2 stage (38.2) with p-value 0.317. (Table 2, Figures 2, 3 and 4)

The mean of ki-67 expression was higher (52.4) in cases that showed positive LVI as compared to those that showed negative LVI (48.0) with p-value= 0.534 as calculated with paired-T test. Cases with positive PNI showed lower mean of ki-67 expression (46.8) than those with negative PNI (51.2) with p-value=0.573.

4. Discussion

This study was conducted on 50 surgically resected specimen of histologically confirmed oral squamous cell carcinoma (OSCC) to compare the expression of ki-67 and the various histopathological prognostic factors of oral squamous cell carcinoma.

Out of 50 studied patients, majority 15 (30%) were 41-50 years of age followed by 13 (26%) in 31-40 years of age group with mean age 48.4±14.4 years of patients. These findings were similar to another North Indian study conducted by Sharma P et al[15] revealed a male to female ratio of 2.2:1 with the largest number of OSCCs developing in the 4^{th} and 5^{th} decades of life. This is also consistent with an earlier report by Mehrotra R et al[16] which concluded that the commonest age of presentation was 5^{th} decade of life. However, the incidence of oral SCC in persons under the age of 50 is increasing.

In the present study, we found male cases (72%) of OSCC outnumbered females (28%) OSCC cases. Male to female ratio was 2.5:1 which is consistent with other north Indian study from U.P on oral carcinoma conducted by Singh MP et al[17] who also observed 3:1 male to female ratio with mean age 48.35 ± 13.07 years of OSCC cases. Socio-cultural norms and values favour easy availability of tobacco products to males.

Tobacco contains many carcinogens which makes oral cavity more vulnerable to cancer. Amount of tobacco consumption is directly proportional to early occurrence of carcinoma. Most common tumor site in this study was buccal mucosa [46%] followed by tongue [44%]. These findings are consistent with study done by Singh MP et al where buccal mucosa and gingivobuccal sulcus were the most affected sites both in males (49%) and females (40%) followed by alveolus which was 25% and 31% respectively. These finding are also consistent with other studies as studied by Mehrotra R et al[18] and Sharma P et al[19] Placement of tobacco quid in the gingivobuccal sulcus region has been attributed to the development of carcinoma. Tongue is the most common site for intraoral cancer among European and the US populations while buccal mucosa to be the most common site among Asian population.

Tongue and floor of mouth carcinoma are more common in western countries due to consumption of alcohol and smoking. The ventral surface of the tongue and the floor of the mouth are the sites most commonly affected by SCC as they are lined by thin non-keratinised epithelium. The carcinogens readily penetrate this thin epithelium to reach the progenitor cell compartment and carcinogens, particularly tobacco products and alcohol in solution, constantly accumulate in the floor of the mouth and bathe the tissues of the floor of the mouth and the tongue.[20]

In our study, majority of tumors were well differentiated squamous-cell carcinoma (WDSCC) 39.2% followed by moderately differentiated squamous-cell carcinoma (MDSCC) 36.7% & poorly differentiated (PDSCC)

Table 2: Association of pathological features with Ki-67 expression

Characteristics		% distribution	Ki-67 expression Mean ±SD	P-value
Histological grade	WDSCC	32	44.2±23.9	0.047
	MDSCC	13	56.5±23.7	
	PDSCC	5	69±18.1	
pT stage	pT1	13	56.2±25.1	0.733
	pT2	21	47.6±25.4	
	pT3	11	49.9±26.0	
	pT4	5	43.9±18.6	
Lymph node status	Node positive	20	50.2±25.8	0.933
	Node negative	30	49.6±23.1	
Lympho- vascular invasion	Present	44	48.0±24.3	0.534
	Absent	56	52.4±25.1	
Perineural invasion	Present	28	51.2±22.4	0.573
	Absent	72	46.8±29.8	

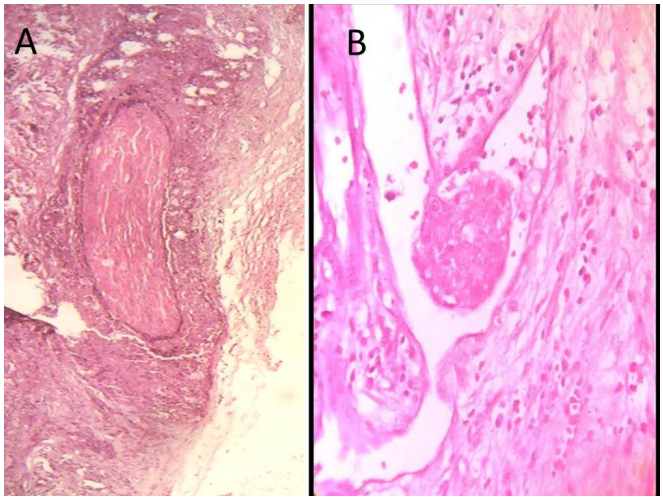

Fig. 1: A): Perineural invasion by Tumor (H&E 10X); **B):** Lymphocascular invasion by Tumor (H&E 10X)

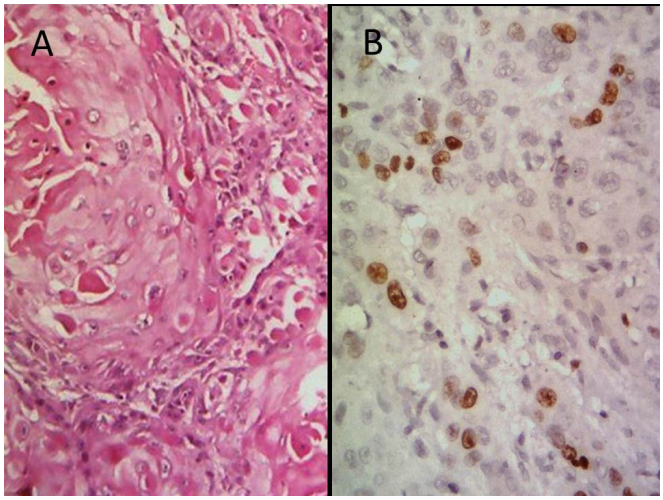

Fig. 2: Well differentiated squamous cell carcinoma; **A):** H&E 40X; **B):** Ki-67 IHC 40X

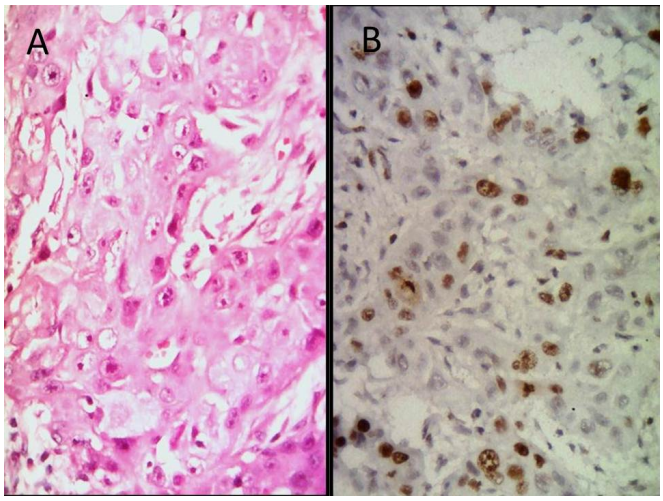

Fig. 3: Moderately differentiated squamous cell carcinoma; **A):** H&E 40X; **B):** Ki-67 IHC 40X

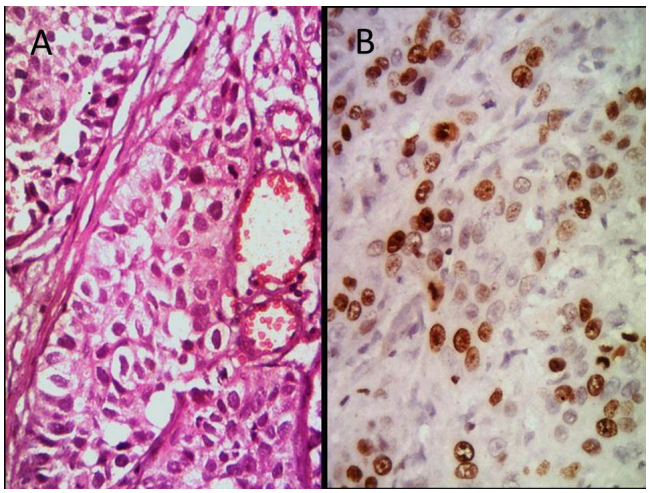

Fig. 4: Poorly differentiated squamous cell carcinoma; **A):** H&E 40X; **B):** Ki-67 IHC 40X

squamous-cell carcinoma 24.1%. In an Indian study, Thiagarajan S et al[21] also reported majority of the patients had a well-differentiated squamous-cell carcinoma (77%). Similarly findings are consistent with the study done by M.A. Gonzalez-Moles et al. In their study, majority of patients 39.2% were of well differentiated followed by 36.7% of moderate ly and least 24.1% of poorly differentiated carcinomas. Another study by Montoro JR et al observed similar trend in the histologic grades of OSCC with most common being WDSCC (51%), followed by MDSCC (47%) and PDSCC (4%).

Distribution of the cases according to pathological-T stage was also observed in the present study which showed maximum cases in pT2 stage (42%) followed by pT1 (26%), pT3 (22%) and pT4 (10%) which was comparable to the study done by M.A. Gonzalez-Moles et al on 79 cases of OSCC who observed 35.5% cases of pT2, 32.9% pT1 and 31.6% cases of pT3 and pT4 combined. Likewise, Montoro JR et al[22] observed on 45 cases, the maximum in pT1 stage (48%) and minimum cases in pT4 (13%). Other studies such as done by Adel M et al[23] observed maximum cases in pT1 and pT2 stage(53%) followed by pT3 and pT4 stage(47%) similar to Franchi A et al[24] who observed maximum cases of pT1 and pT2(62%) collectively, followed by pT3 and pT4(39%) similar to the present study with 68% cases in pT1 and pT2 stage and 32% in pT3 and pT4 stage.

In the present study the distribution of cases according to pN staging was also observed with maximum cases in pN0 stage (60%), followed by pN1 (26%) and pN2 (14%) which was comparable to studies done by M.A. Gonzalez-Moles et al, Adel M et al 135 and Franchi A et al who observed more node negative cases (62.7%, 65.6% and 67.3%) than the node positive cases (37.3%, 34.4% and 32.6%) respectively.

In our study, LVI was identified in 22 patients (44%), Jardim et al found that LVI was present in 40.8% cases of advanced stage in oral squamous cell carcinoma. LVI incidence in present study was comparable with some previously published articles such as published by KA Kurtz et al, who found LVI in as much as 42% cases in a study done on 40 cases of Oral cavity SCC when stained with CD31.[25] Presence of LVI is associated with nodal metastasis and locoregional recurrence.[26] Jones et al[11] reported the incidence of LVI in 69 patients with oral cavity carcinoma to be 35 %.

PNI, in the present study was observed to be present in 28% of cases which was comparable; to the observation in the study conducted by de, Montoro JR et al 134 on 45 patients, in which PNI was found to be positive in 24.44% of cases and similar to the study conducted on 272 patients of OSCC by Brandwein-Gensler M et al with 24% PNI positive cases.[27]

In this study the presence of LVI was observed to be significantly correlated with the increasing histological grades of OSCC with p-value 0.003 comparable to the observation by Adel M et al and Jardim JF et al with p-value <0.001 and 0.03 respectively.

Presence of PNI, in this study, was also observed to be significantly correlated with the increasing histological grades of OSCC with p-value 0.001 comparable to the observations by TC Chen et al[28] who found statistical significance between the presence of LVI/ PNI and increasing histologic grade with p-value 0.02.

In this study, the mean of ki-67 LI was observed to be significantly correlated with the increasing histological grades of OSCC with p-value 0.047 which was comparable to the studies done by R Verma 104, MA Gonzalez - Moles and Prem alatha BR et al[29] who also observed a statistically significant correlation with p-values 0.001, 0.008 and 0.006 respectively. Tumuluri V et al[30] observed that there was a positive association between increasing Ki -67 LI and increasing Broders' grade ($P<0.05$), with a well-differentiated tumour having the lowest mean Ki-67 LI ($1549\pm806/mm^2$) and a poorly differentiated tumour having the highest value ($2232\pm771/mm^2$). A similar trend was observed between the mean Ki- 67 LI and Bryne's multifactorial grading system. They concluded that cell proliferation (as measured by the Ki-67 antigen) at the invasive tumor front had a strong positive relationship with histological grading in human oral SCC.

On comparing the ki-67 mean with the absence or presence of LVI and PNI, statistically insignificant results with p-value 0.534 and 0.573 respectively were obtained, in the present study. Whereas Sengupta P et al[31] found statistically significant correlation of LVI and LVI/PNI with ki-67 expression (p-value <0.001) in a study done on 90 cases of head and neck squamous cell carcinomas. Deniz F et al[32] concluded that there was no statistically significant correlation between KI-67 expression and LV I/PNI (p-value 0.681 and 0.278). To the best of our knowledge, no other studies have attempted to look for such a correlation of ki-67 with PNI and LVI in OSCC.

However, Rakha EA et al[33] compared LVI with ki-67 expression (p-value<0.001) and found statistical significance in invasive breast carcinoma. Stojnev S et al found that Ki-67 expression improved the prediction of LVI ($p < 0.05$) and concluded that Ki-67 overexpression is an independent predictor of LVI in Upper urinary tract urothelial carcinoma, indicating the progression of the disease and Krabbe LM et al[34] observed that Ki-67 over expression was significantly associated LVI ($p = 0.005$) in high grade upper tract urothelial carcinomas.

In this study, statistically insignificant correlation was noted between pT stage and ki-67 expression with p-value 0.733 which was comparable to the observation by Gonzalez-Moles M.A et al with p- value 0.532, whereas Lopes VKM et al[35] found statistically significant correlation (p value 0.0174)

In this study, we also found the association between the lymph node status and Ki-67 expression and the association was found to be non-significant (p value 0.933). This finding is consistent with study of Lopes VKM et al (p-value 0.106)and by Gonzalez-Moles M.A. et al (p-value 0.306) found no significant association between Ki-67 expression and lymph node metastasis.

5. Conclusion

1. This study was undertaken to observe the ki-67 expression in oral squamous cell carcinomas and to compare it with histological grade, pathological T stage, lymph node status, presence or absence of LVI and presence or absence of PNI.
2. Ki-67 expression showed an increasing trend with increasing histological grade and the difference was statistically significant.
3. No significant correlation was observed between ki-67 and pathological T-stage, lymph node status, presence or absence of LVI and presence or absence of PNI.
4. Since there is a paucity of literature studying the correlation between Ki-67 and LVI/PNI in oral squamous cell carcinoma, more studies are needed in this unexplored area.

6. Source of funding

None.

7. Conflict of interest

None.

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