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The relationship of proliferating cell density at the invasive tumour front with prognostic and risk factors in human oral squamous cell carcinoma

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BACKGROUND: We hypothesise that the density of proliferating cells at the invasive tumour front (ITF) has a positive relationship with prognostic and risk factors in human oral squamous cell carcinoma (SCC).

METHODS: Tissues from 47 human oral SCC specimens were collected and stained with a monoclonal antibody directed against the Ki-67 antigen using a horseradish peroxidase based two-step immunostaining method. Counting was performed on two parallel sections at the ITF using an image analyser. The Ki-67 labelling index (LI) was determined by measuring the number of nuclei/mm^2 of epithelium.

RESULTS: Our results show that the density of proliferating cells is related to clinical staging, with advanced stage of disease having a significantly higher Ki-67 LI compared with early stage of disease (2111 ± 905 vs. 1908 ± 913; $P = 0.03$). Importantly, this study shows that tumours that have metastasised have a significantly higher Ki-67 LI than tumours where distant metastasis was not detected (3257 ± 650 vs. 1966 ± 881; $P < 0.0001$).

CONCLUSIONS: Cell proliferation, as measured by the Ki-67 LI at the ITF, has a positive relationship with clinical staging, tumour thickness, smoking status of the patient and alcohol consumption. Further, we suggest that a multicenter study with a large cohort of patients is indicated to fully elucidate whether cell proliferation at the ITF is directly related to patient survival.

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Keywords: cell proliferation; Ki-67 antigen; oral squamous cell carcinoma; staging; prognosis; metastasis

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Introduction

Oral squamous cell carcinoma (SCC) is a malignant neoplasm arising from the mucosal epithelia of the oral cavity. It represents the third most common form of malignancy in developing countries, whilst in developed countries it is the eighth commonest form of malignancy (1, 2). Oral SCC is recognised as a disease resulting from genetic damage, leading to uncontrolled cell proliferation of damaged cells (3). The risk factors for oral SCC include smoking (4) and harmful levels of alcohol consumption (5). The variable histological features of oral SCC are well characterised, and morphological appearance remains the mainstay of diagnosis and prognosis. In contrast, abnormal cell proliferation is considered a central defining feature of all oral SCC but the quantitation of this parameter and its reliability in the clinical situation remains controversial (6, 7)

Cell proliferation is defined as 'increase in cell number resulting from completion of the cell cycle' (8). Many different methods have been developed to detect cell proliferation in malignancies. These methods differ not only in what they detect but also in the phases of the cell cycle that they label the cell. Early methods included mitotic figure counts, S phase labelling with thymidine and Brdu and more recently flow cytometry and *in situ* hybridisation. Variable results between studies or technically cumbersome methods mitigated against the widespread acceptance of these methods. Currently, there is much interest in the clinical utility of immunohistochemical (IHC) markers of cell proliferation, particularly the nuclear markers (9). The two most common IHC nuclear markers used are proliferating cell nuclear antigen (PCNA) and the Ki-67 antigen.

Overall, intraoral SCC has a relatively poor prognosis, with a 5-year survival rate of 35–50% (10, 11). Soames & Southam (12) stated that the most important factor that affects patients' survival is early diagnosis such that the earlier the tumour is diagnosed the better the patient survival. Common poor prognostic factors for oral SCC include grading (13), clinical staging (14, 15), presence of distant metastasis (M) (16), depth of tumour (tumour thickness) (13), site, age of presentation and neural involvement of tumour (17).

Although higher levels of cell proliferation have been detected in oral SCC (18, 19) compared to normal tissue, it has not been yet shown to have any relationship with prognostic factors (6, 19).

Recent seminal studies by Bryne et al. (20, 21) suggest that the invasive tumour front (ITF) is the most prognostic region of the tumour. This finding led us to explore the relationship between histological grading and cell proliferation in oral SCC (18). In this paper, we established that a digitally derived Ki-67 labelling index (LI) from the ITF was directly related to the grading systems currently in clinical use. However, tumour grading is but one of the many factors used to establish the prognosis of oral SCC for an individual. To the best our knowledge, no study to date has explored the relationship between prognostic determinants and a digitally derived Ki-67 LI at the ITF in human oral SCC.

Therefore, the aim of this research is to quantitate the proliferating cell density at the ITF of oral SCCs and to analyse the relationship, if any exists, between the cell proliferation (as expressed by the Ki-67 antigen) LI, with some of the known prognostic factors (namely, staging and tumour thickness) and risk factors (namely, alcohol consumption, smoking history and age).

Materials and methods

The inclusion criteria, immunohistochemical staining method and quantitative analysis of tumours have been published in a previous article (18). Briefly, 47 tumour paraffin-embedded blocks for this research were collected from the Royal Prince Alfred Hospital (RPAH; NSW, Australia) and Westmead Hospital (NSW, Australia) after the relevant ethical approvals were obtained. The inclusion criteria for these tumours were that the lesion should be a primary tumour arising intraorally. Three tissue sections from each tumour were stained for the Ki-67 epitope (mouse monoclonal IgG_1 antibody against the Ki-67 protein, 1 : 100 dilution, Novocastra, Newcastle upon Tyne, UK) using a Horseradish Peroxidase-based two-step immunostaining method (Dako Corp., Carpinteria, USA). Prior to staining, the sections underwent one cycle of a microwave antigen retrieval procedure. The excision margins on the tumour sections were used as the controls. Quantitative analysis of the Ki-67-positive tumour cells was carried out at the ITF (defined as the deepest invasive margin of the tumour histologically) and performed using an image analyser with the help of a macro developed specifically for this study. The counting was performed on two parallel sections where a maximum of 10 Fields of View (FoV) for each slide at a magnification of $\times 200$ ($\times 20$ stage lens, $\times 10$ eyepiece). The Ki-67 LI was expressed as the number of positive cells/mm^2 of epithelium. Statistical analysis was carried out on JMP (version 3.1.6., SAS Institute Inc., NC, USA) and differences were considered significant if $P < 0.05$ using the Tukey–Kramer honestly significant difference (HSD) test. When there was more than one variable post hoc tests were also performed to test for statistical differences.

Clinical and medical data were retrieved from the patients' records. The data collected included: age of the patient at diagnosis, gender, clinical staging, smoking and drinking history of the patient and any serious illness present. The data were collected after the quantitative analysis of the positive cells was complete to ensure that the data did not influence the counts. In a small number of the specimens, the clinical and medical data were incomplete. The clinical stage and the tumour, node & metastasis (TNM) staging recorded by the surgeon at the time of diagnosis were used in this study.

Results

The results showed that a dilution of 1 : 100 of the monoclonal antibody specific for the Ki-67 epitope produced a distinct nuclear immunostain with minimal background staining, and that in 100% of tissue sections, both the tumour front and the excision margin (control) were positive for the Ki-67 epitope. An example of a typical Ki-67 immunostain at the ITF is shown in Fig. 1. An evaluation of the staining distribution showed that the immunopositive nuclei were heterogeneous within the tumour section of each slide. However, the pattern of distribution of the immunopositive nuclei in the excision margin was much less variable. Thus, positive nuclei were restricted to the basal and suprabasal cell layers of the excision margin regions. This contrasted strongly to the tumour region where groups of positive nuclei were highly concentrated in some areas, while other areas were devoid of positive nuclei. However, in general there were more positive nuclei at the ITF than in other areas of the tumour.

The results indicate that the mean Ki-67 LI is significantly higher in the tumour front than at the excision margin (control) tissue for all samples of head and neck SCC (1958 ± 919; 95% CI 1874–2043 vs. 396 ± 194; 95% CI 349–442, $P < 0.0001$; not shown in the table).

Risk factors and cell proliferation

A summary of the data is shown in Table 1. For this study, various patient-related data were collected to observe the influence of these factors on the mean Ki-67 LI. These data include alcohol consumption by the patient, smoking history of the patient and patient age and gender. The most important part of these data set is alcohol consumption and smoking history of the patient, which are also considered risk factors.

It was observed that the mean Ki-67 LI was 11.7% higher in patients who were drinkers compared to patients who were non-drinkers or those who were social drinkers ($P = 0.035$). A similar trend was also observed with smokers, with the mean Ki-67 LI being higher in patients who had a smoking history compared with patients who did not have a smoking history ($P = 0.025$).

Patient age for this study was subdivided into two groups: patients who were 40 or younger and patients who were older than 40 years of age. There was no significant difference between the groups, but a trend ($P = 0.52$) towards a higher Ki-67 LI for patients older than 40 was evident. A similar trend was also observed when cell proliferation was compared with the gender of the patient, where females had a 6% higher Ki-67 LI than males had ($P = 0.16$).

Prognostic factors and cell proliferation

Clinical staging for this study was subdivided into two groups: early tumours comprised clinical stages 1 and 2,

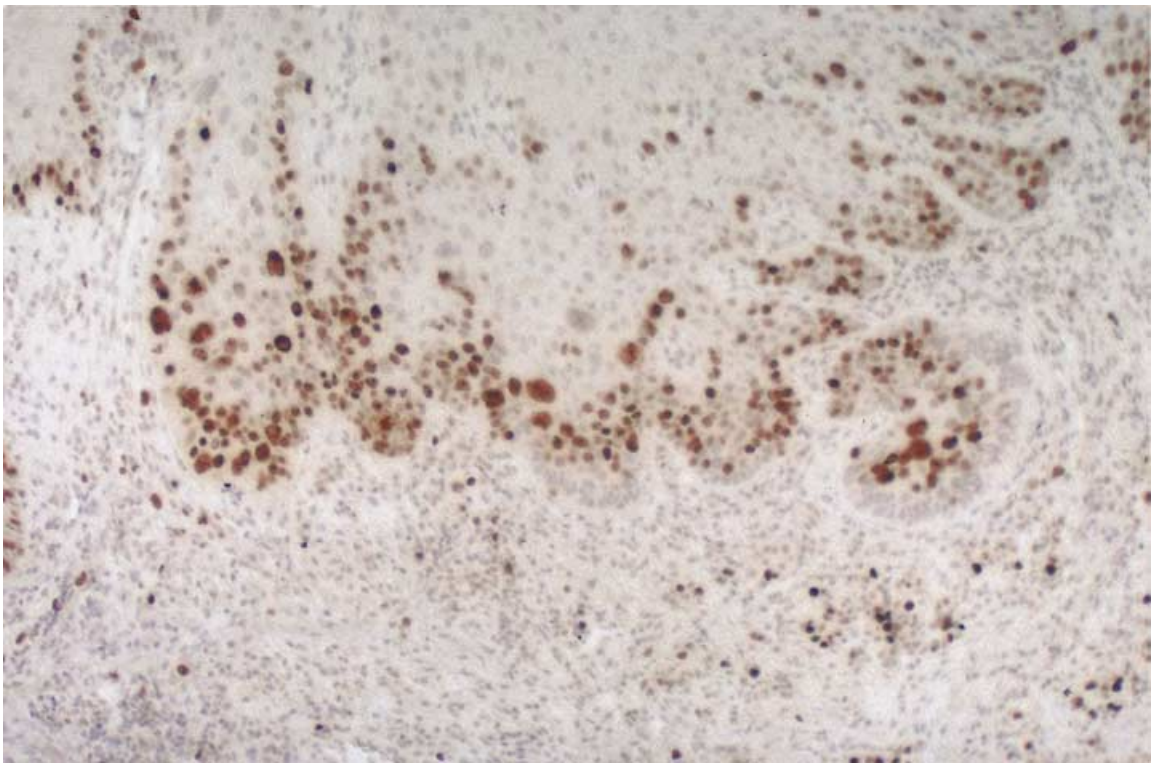

Figure 1 Photomicrograph depicting a typical Ki-67 immunostain at the ITF of oral SCC tissue. DAB (brown stain) was used as the chromogen.

Table 1 A summary of data showing the Ki-67 LI and significance (*P*) value for the different prognostic and risk factors for human oral SCC

	Sample		*Ki-67 LI*		
	n	*Fields of view*	*Mean ± SD*	*95% CI*	*P-value*
Smoking					
−	16	168	1828 ± 824	1702–1953	0.0248
+	17	161	2049 ± 946	1901–2195	
Alcohol consumption					
−	21	215	1861 ± 841	1747–1974	0.0353
+	12	114	2078 ± 966	1898–2257	
Clinical staging					
Early tumours (stages 1 and 2)	18	181	1908 ± 913	1775–2042	0.0341
Late tumours (stages 3 and 4)	20	182	2111 ± 905	1979–2244	
Tumour size (T) (cm)					
<4	21	200	1974 ± 980	1837–2111	0.3614
>4	17	159	2062 ± 803	1936–2187	
Lymph node involvement (N)					
N_0	26	276	2003 ± 849	1902–2103	<0.0001
N_1	7	48	1713 ± 917	1447–1980	
N_2	5	35	2504 ± 1127	2117–2891	
Presence of distant metastasis (M)					
M_0	36	346	1966 ± 881	1873–2059	<0.0001
M+	2	13	3257 ± 650	2864–3650	
Tumour thickness (mm)					
<5	19	170	1838 ± 949	1696–1981	0.0455
>5	22	252	2022 ± 904	1910–2134	
Age					
<40	2	21	1831 ± 543	1599–2069	0.5162
>40	45	434	1964 ± 933	1877–2052	
Gender					
Male	26	244	1902 ± 925	1786–2019	0.1616
Female	21	211	2023 ± 908	1900–2147	

whereas late tumours comprised clinical stages 3 and 4. Our results indicate that the late tumours (clinical stages 3 and 4) had an 11% higher mean Ki-67 LI than the early tumours had (clinical stages 1 and 2; $P = 0.034$).

When cell proliferation was compared with tumour size, it was found that tumours that were greater than 4 cm in size had a 5% higher mean Ki-67 LI compared to tumours that were equal to or less than 4 cm in size, but surprisingly this result was not significant ($P = 0.36$).

When cell proliferation was compared with lymph node involvement, we found that the mean Ki-67 LI in the N_0 tumour group, i.e. tumours without positive nodes was significantly higher than in the N_1 tumour group, i.e. a single positive ipsilateral node less than 3 cm in diameter ($P = 0.03$). However, a tumour that was classified as an N_2, i.e. ipsilateral nodes between 3 and 6 cm or multiple ipsilateral nodes or positive bilateral and contra-lateral nodes, had a higher mean Ki-67 LI than either the N_0 or N_1 had ($P = 0.0017$, $P = 0.0007$).

Cell proliferation was also compared with the presence or absence of distant metastasis, and we found that the mean Ki-67 LI increased by 66% in tumours where distant metastasis was detected in comparison with tumours where distant metastasis was absent. This was statistically significant ($P < 0.0001$).

Our study also analysed the relationship between cell proliferation and tumour thickness. The results showed that tumours where the thickness was greater than 5 mm had a 10% higher mean Ki-67 LI than tumours whose thickness was less than 5 mm. This was statistically significant ($P = 0.045$).

Discussion

To the best of our knowledge this is the first study to report a significant positive relationship between cell proliferation (using the Ki-67 LI) at the ITF and a number of prognostic and risk factors in human oral SCC. This may be because of our improvements to the quantitative method for establishing a reliable LI, the most important features being:

- Method of quantitative analysis: To increase the number of cells counted, we used an image analyser with a macro developed specifically for this study. This also added consistency to our cell counts as compared with manual cell counting and meant that we could count more fields of view (greater area of tumour).
- Selection of field of view: We performed our cell counts at the ITF rather than randomly throughout the tumour. This is based on the evidence by Bryne et al. (20, 21) that the ITF is the most important region of the tumour for diagnostic and prognostic purposes, and further that the entire ITF (as we defined it) was included in our counts.

Cell proliferation and risk factors

In our study, the mean Ki-67 LI was compared with a number of patient-related factors that include smoking history, alcohol consumption and age and gender of the patient. The most important of these were smoking history and alcohol consumption. We have shown that the mean Ki-67 LI for patients who had a past smoking history was significantly higher than that for patients who had no past smoking history. Furthermore, the mean Ki-67 LI for patients who consumed harmful levels of alcohol was significantly higher than patients who were social drinkers or patients who did not consume any level of alcohol. Bundgaard et al. (22) concluded from their study that alcohol and tobacco consumption was associated with poor prognosis for patients with intraoral SCC. Thus, we suggest that the poor prognosis may be reflected by increased cell proliferation in these tumours.

Cell proliferation and presence of distant metastasis (M)

Our results show that the mean Ki-67 LI was higher in tumours where distant metastasis was detected as compared to tumours where distant metastasis was not detected. Although the sample number was low for tumours with the presence of distant metastasis, this result was highly significant. These results are contrary to those provided by Roland et al. (6), who found no significant relationship between the Ki-67 expression and the presence of distant metastasis. It may be implicated from the results of this study that tumours with a high cell proliferation index (such as the mean Ki-67 LI) may indicate a potential for these tumours to metastasise.

Cell proliferation and tumour thickness

The mean Ki-67 LI for tumours that had an infiltrating depth of >5 mm was significantly higher than tumours that had an infiltrating depth of ≤ 5 mm. To the best of our knowledge, our study appears to be the only one comparing the tumour thickness with the Ki-67 antigen expression in human oral SCC. Therefore, meaningful comparisons of LIs cannot be made with other studies.

Cell proliferation and clinical staging, tumour size (T) and lymph node involvement (N)

Our results further indicate that there is no significant difference between proliferating cell numbers at the ITF and the size of the tumour (T). This result is consistent with that provided by Roland et al. (6), who studied Ki-67 expression on frozen sections of oral SCCs, and with that provided by Piffko et al. (19) who studied MIB-1 (a Ki-67 equivalent) LI at the ITF of human oral SCC. The results for tumour size from other studies (6, 19) and the present study may be explained by the fact that the quantitation of cell proliferation in our study was performed at the invasive margin of the tumour. Tumour size (T) is measured on the superficial surface of the tumour, as this is the most accessible surface. Therefore, the size of the proliferative compartment at the invasive margin of the tumour may not reflect the tumour size.

When the density of proliferating cells was compared with lymph node involvement, we found that the highest mean Ki-67 LI was observed for N_2 tumours. Furthermore, the mean Ki-67 LI for N_0 tumours was significantly higher than that for N_1 tumours. These results were significant at the $P < 0.05$ level. Other studies have found no significant relationship between cell proliferation and lymph node involvement (6, 19), which are in contrary to the results of the present study.

Our results indicate that there is a greater density of proliferating cells at the invasive tumour margin of N_0

tumours (i.e. no clinically detected lymph node metastasis) than of N_1 tumours (a single ipsilateral lymph node less than 3 cm detected). Upon cursory examination, this result seems contradictory to the clinical experience. A number of factors might explain this apparent anomaly:

- Recent evidence (23) has suggested that clinical evaluation of neck spread is notoriously unreliable and that a new procedure, sentinel node biopsy, may improve the accuracy (24) of diagnosis of lymph node involvement in the head and neck region (25). An implication that may be suspected from this evidence is that N_0 tumours may have lymph node spread that cannot be detected clinically.
- The sample number of N_1 ($n = 7$) tumours seems to be low in comparison to N_0 ($n = 26$) tumours. This may have had an influence on the mean Ki-67 LI.

A prospective study with a large cohort of specimens might give a more definitive result on the relationship between cell proliferation and lymph node involvement (N) in oral SCC.

Unfortunately, patient survival data are not available at this time for patients used in our study, therefore, comparisons regarding cell proliferation and patient survival cannot be made.

Conclusions

It can be concluded from the results of this study that cell proliferation (using the Ki-67 LI) at the ITF has a direct relationship with clinical staging, tumour thickness, smoking and harmful levels of alcohol consumption by the patient. However, cell proliferation in this study is not related to tumour size and age or gender of the patient. Although results with lymph node involvement (N), presence of distant metastasis (M) and cell proliferation were statistically significant in the present study, these observations have to be interpreted with caution as the sample numbers were low. Whether the Ki-67 LI at the ITF of human oral SCCs has any relationship with patient survival needs to be further elucidated. We believe that the weight of evidence suggests that a large multicentre prospective trial with a large cohort of patients be set up in order to determine if cell proliferation at the ITF has a relationship with patient survival.

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