

Proliferating cell nuclear antigen (PCNA) expression in oral squamous cell carcinoma - an aid to conventional histological grading?

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Abstract:

Proliferating cell nuclear antigen (PCNA) is a well known marker for cell proliferation. It tends to accumulate in the late G₁ and S-phase of the cell cycle. A monoclonal antibody (MoAb) against PCNA is now available and it can react with paraffin-embedded specimens. In the present study, PCNA immunohistochemical staining of 36 cases of oral cancer specimens obtained from surgery were investigated. The results showed differing nuclear staining patterns for PCNA in normal, hyperplastic and dysplastic epithelium, early cancer and 3 levels of differentiation for squamous cell carcinoma of the oral cavity. It appears that PCNA can be a useful marker in delineating normal epithelium and hyperplastic epithelium from dysplasia in the oral cavity. The use of PCNA staining may further emphasize the conventional histopathological grading of well-differentiated, moderately-differentiated and poorly-differentiated oral squamous cell carcinoma but is still dependent on basic criteria as observed without immunostaining. PCNA expression for all grades of squamous cell carcinoma are present at the deep, infiltrative margins.

Key words: Proliferating cell nuclear antigen, oral cancer, oral precancer, histopathological grading.

INTRODUCTION

Oral cancer in some Asian countries is the most common type of malignancy. The incidence varies depending on the countries but can be as high as 50% of all cancer cases.¹ The prognosis of oral cancer is dependent on early detection of cancer and precancerous lesions. The choice of the treatment modalities and whether a radical or conservative approach should be considered may also depend to a certain extent on the stage of tumour cell invasion and histopathological grade of the cancer. However, early cancer and the conventional histopathological grades (Broder's) subdivisions of advanced oral squamous cell carcinoma may not give a true picture of the prognosis of the disease.² Biomarkers may be useful in many cases. The search for biomarkers of cancers which can act as prognostic indicators of the disease is now being actively studied and one such marker is the proliferating cell nuclear antigen (PCNA).

PCNA is a known marker for proliferating cells. Also known as cyclin, it is a 36 kD auxiliary protein for DNA polymerase delta.³ It has been widely used in cell kinetic studies because its expression and distribution correlates with cell proliferation and DNA synthesis. PCNA tends to accumulate in the late G₁ and S phase of the cell cycle.^{4,5} A monoclonal antibody (MoAb) against PCNA is now available commercially. This

antibody has the advantage of being able to react with fresh frozen tissues and paraffin-embedded specimens using immunohistochemical procedures.

Studies on PCNA expression in oral squamous cell carcinoma have shown that PCNA has potential as a biomarker for oral cancer.⁶⁻⁹ The current study was carried out to determine whether PCNA can aid in the conventional histopathological grading of oral carcinoma. It was also the purpose of this study to document the pattern of PCNA expression in oral carcinoma and its marginal edge.

MATERIALS AND METHODS

Fourteen specimens of oral squamous cell carcinoma from the Department of Oral Pathology, Oral Medicine and Periodontology, University of Malaya and 20 specimens from the Oral and Maxillofacial Surgery Unit of Tribhuvan University Teaching Hospital, Kathmandu, Nepal and 2 specimens from the Department of Oral Maxillofacial Surgery, Asahi University, School of Dentistry, Gifu, Japan were used for this study. The specimens, which had been fixed in 10% formalin and embedded in paraffin, were sectioned at 4µm thickness and placed on lysine-coated slides. After deparaffinization and rehydration, one section from each specimen was stained with haematoxylin and eosin (H&E). The other sections

underwent microwave heating. These sections on slides were placed in 1% zinc sulphate solution and allowed to reach a temperature of 100±5°C in the microwave oven for 5 minutes. This process was repeated for another 5 minutes and the sections left to cool for 15 minutes. Microwave heating of sections have been shown to improve antigen retrieval in long term formalin-fixed tissues.¹⁰ After this procedure, endogenous peroxidase was blocked by 0.3% hydrogen peroxide in 99% methanol for 30 minutes. The sections were then treated with normal rabbit serum at 1:20 dilution for non-specific background blocking. This was followed by incubation of the sections with monoclonal mouse anti-PCNA, PC10 (Dakopatts, Denmark) at a dilution of 1:20 for 1 hour. The sections were then reacted with biotinylated rabbit

IgG anti-mouse IgG at a dilution of 1:200 for 30 minutes, followed by treatment with avidin-biotin complex for 30 minutes and visualized with 3-3' diaminobenzidine tetrahydrochloride (DAB) containing 0.005% hydrogen peroxide solution. Normal mucosa was used as a positive control and replacement of the primary antibody by PBS as a negative control step.

The histopathological assessment for epithelial dysplasia was based on WHO criteria." The conventional criteria for the different grades of squamous cell carcinoma were based on the Broder's classification.¹² Early squamous cell carcinoma was not graded and was defined as carcinoma where the invasion involved only the lamina propria. These criteria were as listed in Table 1.

TABLE 1: Histological criteria for dysplasia and oral squamous cell carcinoma (SCC)

Nomenclature	Criteria	References
Epithelial dysplasia (no subdivision into mild, moderate or severe)	Presence of some of these features below: 1. Basal cell hyperplasia 2. Irregular epithelial stratification 3. Nuclear hyperchromatism 4. Keratinization of single/groups of cells in prickle cell layer 5. Increased number of mitotic figures 6. Reduction of cellular cohesion 7. Enlarged nucleoli 8. Increased nuclear: cytoplasmic ratio 9. Drop-shaped rete processes 10. Mitotic figures in superficial half of epithelium 11. Loss of polarity of the basal cells 12. Cellular pleomorphism	WHO Collaborating Centre for Oral Precancerous Lesions ¹¹
Oral SCC (Broder's Classification):		
1. Well - differentiated SCC	Relatively mature tumour cells with few nuclear aberrations and with the presence of keratin pearls and/or individual cell keratinization.	Anneroth & Hansen ¹²
2. Moderately-differentiated SCC	The presence of tumour cells exhibiting a wide range of differentiation. Keratinization was occasionally present and nuclear aberrations were moderately abundant.	
3. Poorly-differentiated SCC	Disorderly and poorly differentiated tumour cells with no tendency to keratinization . Nuclear aberrations were abundant.	

RESULTS

Positive staining of nuclei of surface epithelial and cancer cells was observed in all specimens. The number of cells with positive nuclear PCNA immunoreactivity varied in different specimens. Histological examination of H&E sections showed that out of 36 specimens, 8 were early invasive carcinoma, 10 were well-differentiated carcinoma, 8 were moderately-differentiated squamous cell carcinoma and 10 were poorly-differentiated carcinoma. In many specimens, normal, hyperplastic and dysplastic epithelium at the tumour margins were also evident.

In normal epithelium, positive nuclear PCNA staining of the basal cells and few parabasal cells was evident (Figure 1A & B). However, positive nuclear staining was observed in the basal, parabasal and the spinous cell layer of dysplastic epithelium (Figure 1C).

In early invasive carcinoma, positive nuclear PCNA staining was observed in the basal and parabasal layer with very intense nuclear staining of the cells at the infiltrating margins (Figure 2B). Cases of early invasive carcinoma also showed moderate dysplasia of adjacent surface epithelium. Positive nuclear PCNA staining was observed in the basal, parabasal and lower spinous layer of the dysplastic epithelium. Positive nuclear staining of the cancer cells were evident in hyperchromatic nuclei but not in clear/vacuolated nuclei (Figure 2A & B).

All moderately-differentiated carcinomas showed nuclear staining of peripheral cells of the tumour islands containing mature tumour cells (Figure 3B). In areas showing lesser degrees of differentiation (less mature tumour cells), there was variable staining with no particular pattern. There was positive PCNA staining of small infiltrating and invasive nests of tumour cells. There was also positive nuclear staining of the basal cells of adjoining hyperplastic epithelium while intense positive nuclear staining for PCNA was seen in the basal and parabasal cells at the junction of the tumour margin and hyperplastic epithelium.

The well-differentiated carcinomas exhibit positive nuclear PCNA staining of the peripheral cells of the tumour islands (Figure 3C). There was no staining of the keratinized cells or the keratin pearls. Positive PCNA staining was evident within the tumour cells of the invasive/infiltrative tumour islands (Figure 3D). The adjacent surface epithelium exhibited positive staining of some basal cell nuclei.

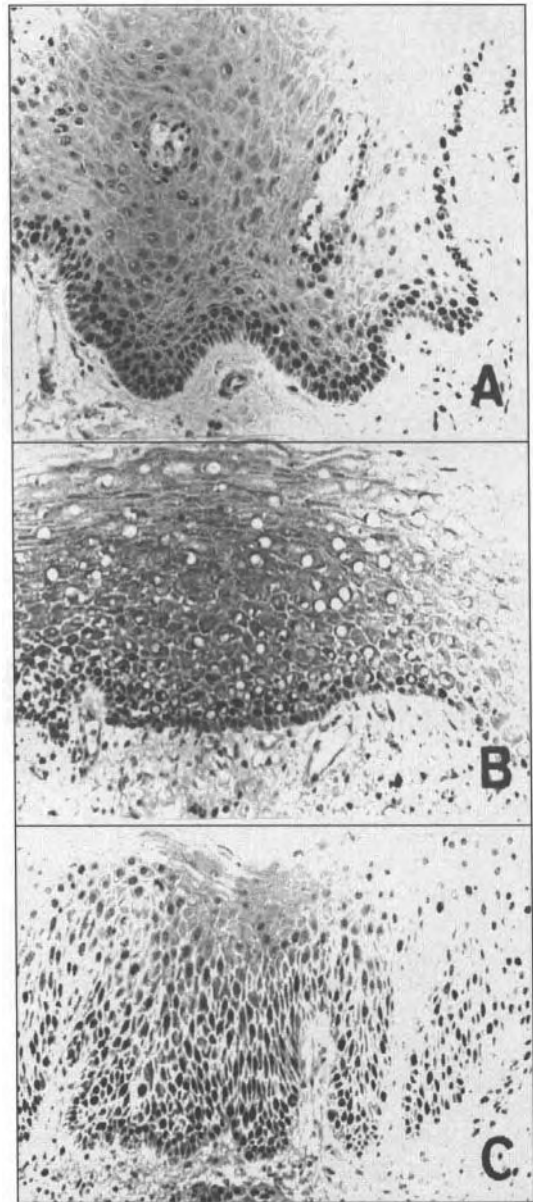


FIG. 1A-B: Normal and hyperplastic oral epithelium, PCNA positive cells in the basal layer; **C:** Dysplastic oral epithelium, PCNA positive cells in the basal and parabasal layers. Original magnification x 100.

A heterogeneity of PCNA nuclear staining was observed in poorly differentiated carcinoma. In all the 10 cases of poorly-differentiated carcinomas, some of the hyperchromatic and anaplastic nuclei of the infiltrating cell nests were intensely stained for PCNA (Figure 4B). Positive nuclear PCNA staining of the infiltrative/invasive tumour cells was evident but clear/vacuolated nuclei in the infiltrative cell nests were negative for PCNA (Figure 4D).

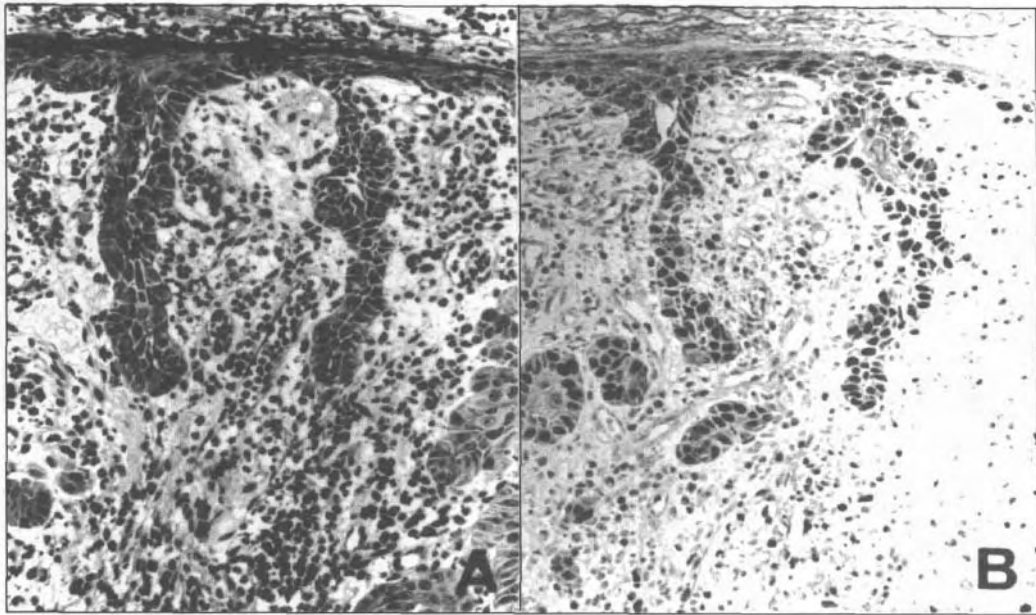


FIG. 2A-B: Early invasive carcinoma; A: haematoxylin & Eosin; B: Intense nuclear PCNA staining at the infiltrating margins. Original magnifications $\times 100$.

DISCUSSION

Qualitative analysis of the characteristic pattern for nuclear PCNA immunoreactivity in normal and hyperplastic epithelium, dysplastic epithelium and the different grades of squamous cell carcinoma in this study was found to be different for each of these entities. These qualitative observations were similar to the studies by Tsuji *et al*^{6,7} and Coltrera *et al*.⁸ The conventional criteria of grading dysplasia had been published in 1978 by the World Health Organisation (WHO) collaborating centre for oral precancerous lesions.¹¹ However, Pindborg *et al*¹³ had shown non-uniformity in assessing the different levels of dysplasia by 72 participants of the 2nd Meeting of the International Association of Oral Pathologists in Amsterdam in 1984. Pindborg *et al* summarized that such an exercise represented the need for uniform criteria for diagnosis of oral epithelial dysplasia. This study and other recent studies have shown that nuclear staining for PCNA in dysplastic oral epithelium involved the basal, parabasal and spinous cell layer as compared to normal and hyperplastic epithelium where the nuclear PCNA staining was only in the basal area.^{6,7,8} Suprabasal staining was observed in all types of dysplasia and carcinoma-in-situ by Coltrera *et al*.⁸ Tsuji *et al*⁶ had quantitated the expression of PCNA and found that the growth fractions were higher in leukoplakia as compared to normal mucosa. However, leukoplakia may have histological characteristics ranging from

epithelial hyperplasia without dysplasia, hyperkeratotic epithelium, varying degrees of dysplasia, early invasive carcinoma or combinations of these histological pictures.¹ In view of this, future studies can be conducted to correlate between the subdivisions of leukoplakia according to histological characteristics and PCNA activity. At this stage many studies, including our current study, show that PCNA is a useful marker in differentiating dysplastic epithelium from normal epithelium.^{5,6,7} However, more investigations will be required to ascertain whether the different grades of mild, moderate and severe dysplasia and carcinoma-in-situ can be differentiated further with the use of PCNA as a biomarker.

Similarly, in the area of early cancer, PCNA immunostaining may be useful in differentiating more aggressive tumours (where nuclear staining for PCNA occurs in many nests of cells at the infiltrating margins) from less aggressive tumours (where this is nuclear staining of only peripheral cells of tumour islands at the invasive margins) with a tendency towards a well differentiated carcinoma. The ability to predict the grade of carcinoma at an early stage may be useful for oral and maxillofacial surgeons in deciding the best treatment for the patient and thus giving a better prognosis for the patient.

In advanced oral carcinoma, a definite pattern of PCNA distribution in well-differentiated, moderately-differentiated and poorly-

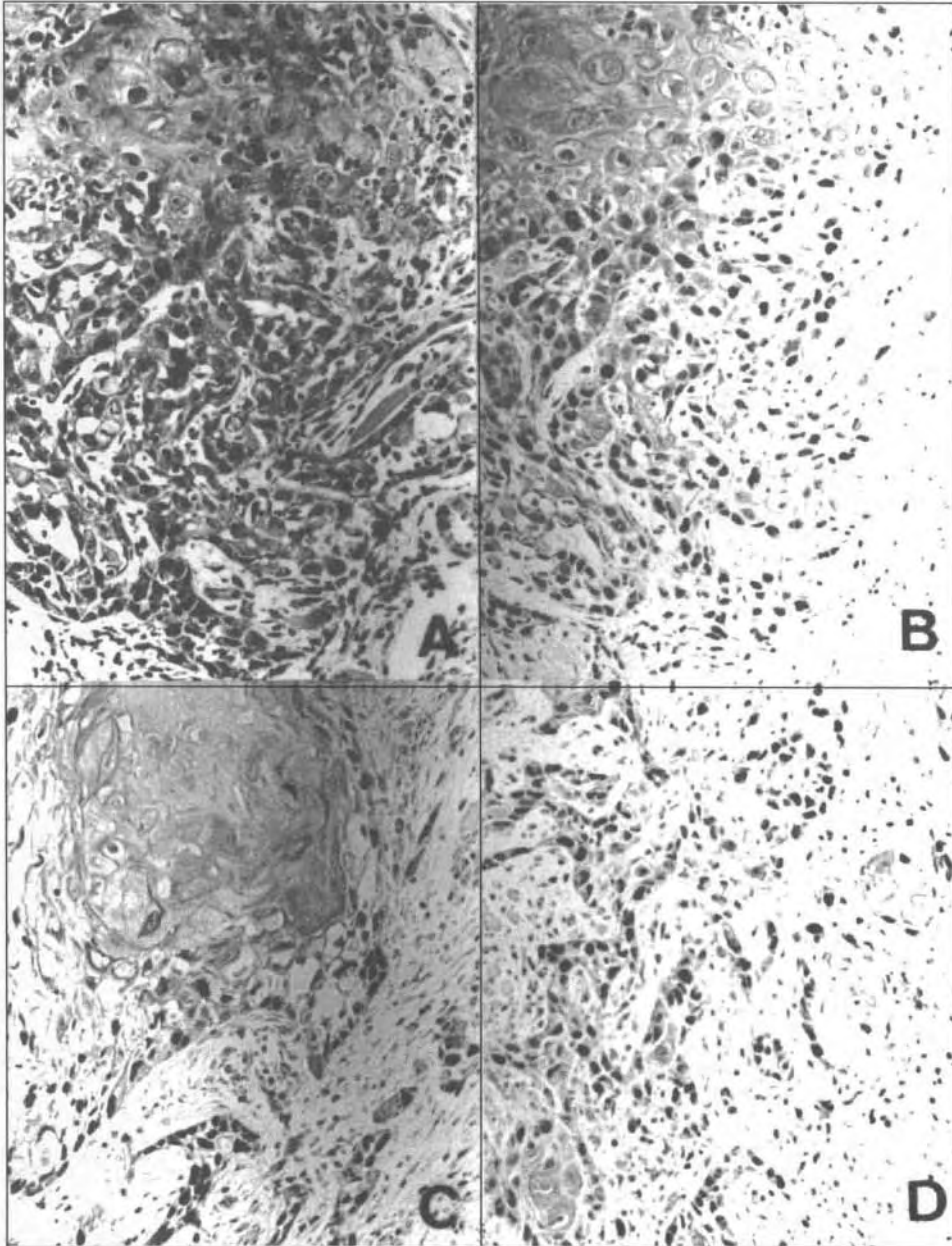


FIG. 3A-B: Moderately-differentiated squamous cell carcinoma; A: Haematoxylin & eosin stain; B: Positive PCNA staining of peripheral cells of the mature tumour islands and positive PCNA staining of the infiltrating nests of cells. C-D: Well-differentiated squamous cell carcinoma; C: Positive PCNA staining of the keratinized tumour islands with no PCNA staining of the keratinized cells. At the invasive border, there is positive PCNA staining of the infiltrating tumour cells. Original $\times 100$.

differentiated carcinoma has been consistently reported by recent studies.^{6,7} This pattern is again reproduced in our study where a well-differentiated carcinoma showed nuclear staining of the peripheral cells of the mature tumour islands, a moderately-differentiated carcinoma showed nuclear staining of peripheral cells of mature tumour islands and variable staining of the less differentiated or mature areas and a poorly-

differentiated carcinoma would exhibit a heterogeneity of PCNA staining. However, even though such a definite pattern of PCNA staining can be observed, its use can only complement the conventional histopathological grading.

Many publications had also indicated that the conventional histopathological grading for squamous cell carcinoma by Broder's was of limited value.^{2,12} These authors found that whilst

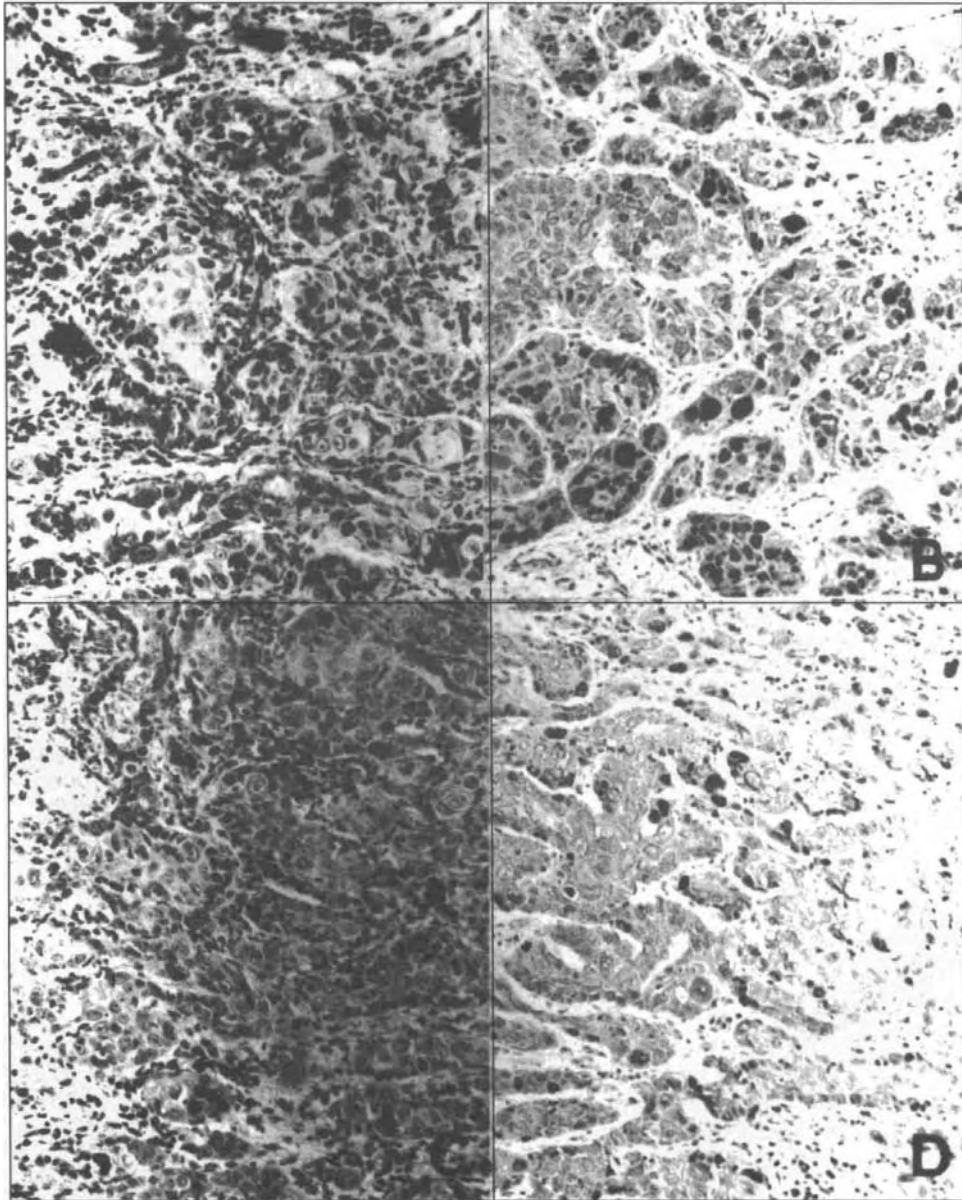


FIG. 4A-D: Poorly-differentiated squamous cell carcinoma. A & C: Haematoxylin and Eosin staining; B & D: Some hyperchromatic and anaplastic nuclei are stained for PCNA. Clear or vacuolated nuclei are negative for PCNA. Original magnifications $\times 100$.

Broder's classification only described the differentiation or maturation of the tumour cell population, the consideration of tumour cells and tumour-host relationship, termed the Malignancy Grading System (MGS)¹⁴ was a better histopathological indicator for prognosis and treatment. A series of development and modification of the MGS have been investigated (Table 2). More recently, it was found that the modified MGS system when applied to the invasive margins, showed better prognostic potential.¹⁵ In this regard, the presence of PCNA

staining in tumour cells at the invasive margins in all conventional grades of SCC in this study supported the latest view that there is a variable proliferation potential in these deep invasive areas.¹⁵ Furthermore, recent work on leukoplakia in our laboratory have also shown different PCNA labelling indices for different types of tissue. There was a higher index for moderate to severe atypia as compared to hyperkeratosis and normal oral mucosa (Table 3). Future studies evaluating the growth potential of oral SCC based on PCNA labelling indices at the deep invasive margins

TABLE 2: Histopathological grading of oral SCC

Grading System	Basis of grading	References
Broder's Classification (1941)	Differentiation/maturation of tumour cell population only.	Anneroth & Hansen, 1984 ¹²
Malignancy Grading System - MGS (1973) Modified MGS (1984)	Point system taking into account both the tumour cell population & tumour-host relationship.	1. Jakobbson et al, 1973 ¹⁵ 2. Anneroth & Hansen, 1984 ¹²
Invasive cell grading (1992)	Point system taking into account both the tumour cell population & tumour-host relationship at the invasive margins only.	Byrne et al, 1992 ¹⁶

TABLE 3: PCNA Labelling Index in Leukoplakia

Specimen	% PCNA positive nuclei (Mean)
Normal oral mucosa	9.52
Hyperorthokeratosis	11.88
Hyperkeratosis	13.01
Hyperkeratosis + acanthosis	15.52
Slight atypia	17.35
Moderate to severe atypia	22.36

Total no. of cases = 114

PCNA labelling index = PCNA labelled cells in at least 1000 cells of leukoplakia.

may be fruitful. We note that PCNA antigen in specimens that had been fixed for up to 48 hours still maintained its reactivity.⁶ This is advantageous in view of the fact that some surgical specimens may have been in fixatives for a long time. The antigen retrieval method using microwave heating as used in this study may further enhance PCNA reactivity by exposing more antigenic sites:

Quantitative assessment of PCNA expression can play important roles in the prediction of long term survival, planning of prophylactic adjuvant therapy and the choice of treatment modalities. It may also serve as prognostic indicators of malignancies and aid in monitoring the progression of treatment. A study on colorectal carcinoma has shown that immunohistochemical analysis may aid in the prediction of long term survival and the planning of prophylactic adjuvant therapy.¹⁶ PCNA labelling rates appeared to be potentially useful prognostic indicators for gastric cancer¹⁷ and in T1 glottic cancer, optimum treatment regimes have been moderated by assessing PCNA

expression.¹⁸ A high PCNA expression would indicate a high proliferative activity in the cancer and therefore the patient would be treated with radiotherapy which was more specific for proliferating cells. In the field of oral cancer, Tsuji et al¹⁷ has shown that the mean PCNA score decreased significantly from 20% to 8% after cancer chemotherapy. They concluded that the measurement of PCNA can be used to monitor and evaluate the success of treatment modalities such as chemotherapy.

Arising from the current and other studies on oral epithelial precancerous and cancerous lesions, it appears that PCNA can be a useful biomarker for delineating normal epithelium from dysplastic epithelium. However, PCNA staining can only supplement the conventional grading of oral SCC as viewed by light microscopy using the haematoxylin and eosin stain.

We have shown that PCNA activity was present in the invasive margins of all the conventional grades of oral SCC. Thus assessments of PCNA

