Epithelial cell proliferative activity and oral cancer progression

P. J. Thomson, J. V. Soames, C. Booth* and J. A. O'Shea*

*Departments of Oral & MaxilloFacial Surgery and, Oral Pathology, Dental School, University of Newcastle upon Tyne., UK *Epistem Ltd, Incubator Building, Grafton Street, Manchester, UK*

Abstract. Accurate, predictive assessment of the behaviour and progression of oral cancers and precancers remains elusive in clinical practice. Archival tissue specimens from 10 previously treated patients with oral lesions of known clinical outcome (3 years post-treatment) were re-examined histopathologically, and proliferative cell labelling indices (LIs) determined for Ki67, cyclin A and histone mRNA cell cycle markers. While histone mRNA labelling ultimately proved unreliable, both Ki67 and cyclin A LIs demonstrated a clear trend for enhanced labelling to occur in increasingly dysplastic and neoplastic tissue, with particular emphasis on suprabasal labelling in abnormal tissue. Perhaps of greatest significance was the observation of increased LIs and suprabasal labelling in lesions with poor clinical outcome, such as patients developing recurrent disease or cervical lymph node metastasis. Measurement of cell proliferative activity in individual oral epithelial dysplastic lesions or invasive squamous cell carcinomas may thus provide unique, predictive information on clinical outcome.

Keywords: clinical outcome, cyclin A labelling, histone mRNA, Ki67 labelling, oral cancer, oral dysplasia, oral squamous cell carcinoma.

INTRODUCTION

Oral carcinogenesis is believed to be a multistep process derived from an accumulation of cellular alterations induced by carcinogens. Fundamental to this process are the alterations in genes regulating cell division, cell cycle progression and DNA synthesis and repair. The results of these molecular and genetic alterations are phenotypic changes in epithelial tissue, which may be recognized histologically as epithelial dysplasia, ultimately representing dysregulation of cell proliferation and differentiation (Kushner *et al.* 1997; Gonzalez-Moles *et al.* 2000).

It is believed that the cells most likely to be associated with premalignant change during carcinogenesis are those with the highest proliferative activity, and that increasing deregulation of cell proliferation plays a central role in tumorigenesis (Jordan *et al.* 1998). Unfortunately, accurate, predictive assessments of the clinical behaviour and progression of individual oral cancers and precancers remains elusive in clinical practice.

In previous studies we used epithelial cell proliferative activity, measured by S-phase labelling indices to quantify both dysplasia and malignancy and have confirmed increased labelling with worsening dysplasia and decreased tumour differentiation (Thomson *et al.* 1999). Although

interesting research tools, these labelling techniques are disadvantaged by their requirement for immediate *in vitro* incubation of freshly excised oral mucosa, time-consuming laboratory processing and demanding analyses and cell counting. Most significantly, measurement of clinical outcome remains elusive, requiring years of patient follow-up to establish. Alternative labelling methods, however, are available that are applicable to archived, formalin-fixed histological specimens in which clinical outcome is known and documented.

The cell cycle and its control is known to be complex, but improved understanding of the molecular events governing specific cycle events and the transition between phases has led to improved diagnostic and labelling techniques, many of which can now be applied to archived, formalin-fixed tissue specimens. Similarly, alteration in cyclins (the proteins regulating the passage of dividing cells through critical cycle checkpoints) are being increasingly identified in a variety of human tumours as important contributors to increased cell proliferation (Kushner *et al.* 1999).

The aim of this pilot study was to use specific markers of cell cycle activity to examine cell proliferation within treated oral cancer or precancer lesions with known clinical outcome and to determine their potential usefulness as predictors of clinical behaviour.

Three markers were used: (1) Ki67 antigen, (2) cyclin A and (3) histone mRNA. Ki67, a proliferation-associated protein active in dividing cells throughout the cycle and used to estimate the growth fraction of a tissue, and cyclin A, a cyclin protein synthesized primarily during S phase, required for both S phase progression and passage of cells from G2 into mitosis, are both detectable in formalin-fixed tissue specimens using immunohistochemistry, while histone mRNA, synthesized during S phase and rapidly degraded following DNA replication, is detected by *in situ* hybridization.

METHODS

Following local Ethical Committee approval, 10 previously treated oral cancer or precancer patients were randomly selected from the Oncology database of the University of Newcastle Department of Oral and Maxillofacial Surgery. Inclusion criteria required a first presentation of a dysplastic or neoplastic lesion, with no previous history of oral cancer, surgical treatment or radiotherapy, with all details of clinical outcome, disease recurrence or further disease development recorded during a 3-year follow up period.

Formal histopathological re-examination of archive material was carried out; routinely processed, paraffin-embedded haematoxylin and eosin-stained sections were reviewed to ensure that sufficient, well-orientated tissue was available for study. Dysplastic lesions were graded subjectively as mild, moderate or severe; mild dysplasia was defined as cellular atypia confined to the basal third of the epithelium, moderate involved up to two-thirds and severe, greater than two-thirds. Invasive squamous cell carcinomas (SCCs) were graded conventionally into well, moderately or poorly differentiated tumours according to established criteria.

Immunohistochemical detection of the cell proliferation-associated antigen Ki67 (NCL-Ki67-MM1; Novocastra, Newcastle, UK) and of cyclin A (NCL-CYCLIN A; Novocastra) was performed using a standard avidin-biotin-peroxidase technique following high-temperature, pressure-cooker antigen unmasking in 10 mM citrate buffer (pH 6.0).

Histone mRNA was localized by *in situ* hybridization using a fluorescein-conjugated histone probe (NCL-HISTONE-513; Novocastra). Bound probe was detected with an anti-FITC-AP antiserum and visualized by addition of an enzyme substrate.

Table 1. Clinical details of patients

Age	Sex	Site	Clinical diagnosis	Histopathology	Treatment	Clinical outcome
50	F	R lateral tongue	SCC	Mod. diff. SCC	Surgical excision	Second primary (R tonsil)
57	F	L lateral tongue	SCC	Mod. diff. SCC	Surgical excision	Cervical metastasis
71	F	L ventral tongue	Leukoplakia	Moderate dysplasia	Laser excision	No recurrence
73	F	L buccal mucosa	Erythroleukoplakia	Moderate dysplasia	Laser excision	Local recurrent disease
76	F	R ventral tongue	Leukoplakia	Cellular atypia	Laser excision	No recurrence
77	F	L ventral tongue	Leukoplakia	Severe dysplasia	Laser excision	No recurrence
53	M	L lateral tongue	Leukoplakia	Micro-Invasive SCC	Laser excision	Died. Second primary (larynx)
57*	M	L floor of mouth	Normal Mucosa	'Normal'	None	No disease*
58	M	R floor of mouth	Leukoplakia	Moderate dysplasia	Laser excision	No recurrence
59	M	R buccal mucosa	Erythroleukoplakia	Severe dysplasia	Laser excision	No recurrence
81	M	L floor of mouth	SCC	Mod. diff. SCC	Surgical excision	No recurrence

*Control patient. Mod. diff., moderately differentiated.

Following tissue processing, samples were analysed using a computer-assisted microscope system that functions by superimposing the computer screen on to the microscope image (Zeiss AxiohomeTM interactive microscope;). The base of five successive epithelial ridges or five representative fields of view, depending upon tissue type and organization, were defined for each sample and displayed on both computer screen and microscope eyepiece simultaneously. Various parameters associated with the defined tissue were calculated by computer: area studied, the length of the basal layer and overall tissue thickness. Further assessment identified cells by their labelling characteristics and/or location, allowing determination of percentage labelling indices, structural hierarchy, and the number of cells per unit area or unit length.

RESULTS

Ten suitable patients were identified: six female (age range 50–77 years, mean 67.3 years; presenting as two oral SCCs and four dysplastic leukoplakias) and four male (age range 53–81 years, mean 62.75 years; presenting as one oral SCC, one microinvasive carcinoma and two dysplastic leukoplakias). One case control was included in the study: a 57-year-old-male, a known tobacco and alcohol user requiring excision of clinically normal mucosa from the floor of the mouth during ranula removal. Table 1 summarizes these details, together with the relevant management and clinical outcome for each case: in seven (six patients plus control) no recurrent or further disease was identified, in one local disease recurred, two exhibited new disease (second primaries in the tonsillar fossa and larynx) and one developed cervical lymph node metastases.

Histological examination of tissue specimens confirmed successful and consistent labelling for both Ki67 and cyclin A with excellent preservation of tissue morphology (Figs 1 and 2). Unfortunately, histone mRNA labelling proved to be unreliable, with only 3/11 sections staining effectively (Fig. 3); the majority of histone slides displayed patchy staining and tissue damage.

Figure 1. Photomicrograph of Ki67-labelled dysplastic buccal mucosa erythroleukoplakia ($\times 22$).

Table 2 summarizes tissue labelling indices and morphological analysis (cells/unit length or area) for the 11 subjects recruited. No demonstrable relationships were evident between patient age, sex or site of lesion and tissue labelling indices, but this is probably unsurprising in view of the small number of samples studied.

Comparison of Ki67 and cyclin A labelling indices with histopathological diagnosis demonstrated a clear relationship between increased LIs and increasing dysplasia and neoplasia (Figs 4a and 5a), although the poor quality of labelling with histone mRNA made a similar relationship less clear (Fig. 6a). Topographical analysis reveals a trend for increased suprabasal labelling in more severely dysplastic and neoplastic tissues (Figs 4b, 5b and 6b).

Four categories of clinical outcome were delineated for patients during the study period: no recurrent disease, the development of recurrent/further disease at the same anatomical location, new disease at different oral sites, or the development of cervical lymph node metastases. The highest Ki67 labelling indices were observed in the three 'further disease' categories (Fig. 7a), while suprabasal Ki67 labelling was also more marked in these categories (Fig. 7b). Similar results were seen with cyclin A labelling (Fig. 8a,b), but histone mRNA labelling was unreliable (Fig. 9a,b).

Analysis of the SCC samples was inevitably complicated in parts of the sections studied by lack of normal epithelial architecture, but the use of five representative fields of view for cell scoring helped to establish consistent, overall LIs.

Table 2. Tissue labelling details

Histopathological diagnosis	Tissue source	Total LI (%)			Basal LI (%)			Suprabasal LI (%)			Mean cells/ unit length (mm)	Mean cells/ unit area (mm ²)
		Ki67	Cy. A	Histone mRNA	Ki67	Cy. A	Histone mRNA	Ki67	Cy. A	Histone mRNA		
Normal	Floor of mouth mucosa	15.8	4.8	0.0	7.6	1.9	0.0	20.9	6.6	0.0	138.4	9 841
Cell atypia	Ventral tongue leukoplakia	14.2	7.4	0.1	17.9	8.3	0.0	13.5	7.2	0.2	116.2	4 355
Moderate dysplasia	Ventral tongue leukoplakia	22.2	13.3	10.9	14.7	10.4	6.7	25.9	14.4	13.3	124.6	9 487
Moderate dysplasia	Buccal mucosa erythro-leukoplakia	37.2	11.4	0.0	14.9	6.6	0.0	45.8	13.0	0.0	134.0	9 968
Moderate dysplasia	Floor of mouth leukoplakia	22.7	9.6	0.0	16.4	13.3	0.0	24.8	8.2	0.0	134.7	10 427
Severe dysplasia	Ventral tongue leukoplakia	18.8	10.4	0.2	29.8	21.7	0.0	15.1	7.2	0.3	98.2	2 905
Severe dysplasia	Buccal mucosa erythro-leukoplakia	27.9	12.9	6.6	31.1	15.0	8.6	26.6	11.7	5.8	101.0	6 359
Micro-invasive SCC	Lateral tongue leukoplakia	29.1	11.6	0.1	21.8	7.0	0.4	32.0	13.3	0.0	115.4	7 874
SCC	R lateral tongue SCC	32.6	17.0	0.2	38.5	17.4	0.0	30.9	17.1	0.3	119.0	5 227
SCC	Left lateral tongue SCC	39.5	17.2	12.4	14.7	10.4	4.2	49.2	20.1	16.5	121.5	9 924
SCC	Floor of mouth SCC	27.2	7.1	0.7	10.1	4.6	0.0	35.0	8.41	1.0	138.9	10 862

Cy. A, cyclin A; LI, labelling indices.

DISCUSSION

The management of established oral squamous cell carcinoma (OSCC) may require extensive tumour resection and surgical reconstruction, often combined with radiotherapy. Post-treatment morbidity is high, and the disease is still lethal, with 5-year survival rates less than 50% (Brown & Langdon 1995). New approaches for controlling OSCC are thus important, and must include the earliest possible detection of invasive disease, and the identification of precancerous lesions with definite malignant potential (Lippman & Hong 2001).

Oral premalignant lesions are known to represent an increased risk for cancer development within the mouth, with quoted malignant transformation rates varying from 6.6 to 36% (Oliver *et al.* 2000), but unfortunately no reliable or objective clinical or histopathological features exist to predict behaviour of individual lesions or patients, although it is generally agreed that lesions exhibiting oral epithelial dysplasia (OED) are at increased risk. Increased cell proliferation is a feature of both OED and OSCC and may thus be associated with an increased risk of malignant transformation (Jordan *et al.* 1998; Thomson *et al.* 1999; Oliver *et al.* 2000).

The basal cell layer is the proliferative compartment of normal oral epithelium, but increasing levels of dysplasia may be recognized by the presence of proliferating cells in both suprabasal and superficial cell strata (Kushner *et al.* 1997; Gonzalez-Moles *et al.* 2000; Liu & Klein-Szanto 2000).

Ki67 has been recommended as a reliable and consistent immunohistochemical marker in cell proliferation studies, showing increased expression with both increasing dysplasia and worsening malignancy (Zoeller *et al.* 1994; Kushner *et al.* 1997). Gonzalez-Moles *et al.* (2000) emphasized the importance of recognizing proliferative activity beyond the basal layer and highlighted the value of suprabasal Ki67 expression as an objective marker of OED that increases proportionate to the severity of dysplasia. Our findings were very similar, particularly in relation to the increased suprabasal labelling evident in dysplastic and OSCC lesions (Fig. 4).

Other workers have reported a lack of oral epithelial basal cell staining with Ki67 (Girod *et al.* 1993; Oliver *et al.* 2000), but this did not appear to be the case in our study (mean basal Ki67 LIs ranged from 7.6 to 38.5%, compared with a suprabasal range of 13.5–49.2%; Table 2).

Kushner *et al.* (1999) observed overexpression of cyclin A protein in OSCC and confirmed a strong correlation with Ki67 labelling, suggesting that cyclin A may be a useful measure of increased proliferative activity in tumour tissue. Oliver & MacDonald (2000), however, compared cyclin A labelling with S-phase BrdU LIs in 11 OED lesions and noted that cyclin A LIs were higher than corresponding BrdU levels, particularly for the most dysplastic lesions, raising concern that cyclin A may be intrinsically overexpressed during carcinogenesis.

In the present study, cyclin A LIs were lower than their Ki67 counterparts (Table 2), probably reflecting the predominantly S phase activity of cyclin A compared with Ki67 as an overall proliferation marker; this is similar to the findings of Oliver & MacDonald (2000).

Histone mRNA, however, is synthesized during S phase and rapidly degraded following DNA replication. *In situ* hybridization, a technique analogous with immunocytochemical analysis, was used in an attempt to provide precise determination of S phase LIs. Unfortunately, the use of the NCL-HISTONE-513 probe in this study was not associated with consistent or reliable labelling. Of particular concern was the presence of tissue damage following the use of proteinase K, which facilitated diffuse spread of histone, resulting in nonspecific labelling. While this may have been a technical difficulty due to unfamiliarity with the hybridization technique, it was disappointing not to have been able to analyse these sections further.

Figure 2. Photomicrograph of cyclin A labelled dysplastic floor of mouth leukoplakia ($\times 22$).

Prediction of clinical outcome in both precancer and cancer patients has always been notoriously unreliable, although analysis of lesional DNA content, as recently described by Sudbo *et al.* (2001), may be a promising technique, demonstrating particularly high malignant transformation rates in aneuploid dysplastic lesions compared with diploid counterparts.

Both Ki67 and cyclin A LIs obtained in this study, however, do appear useful as predictors of clinical outcome, with a clear trend for highest LIs to be seen in patients exhibiting recurrent or further oral lesions or malignancies developing cervical metastases (Figs 7a and 8a). Of particular interest is the demonstration of enhanced suprabasal labelling in cases with compromised clinical outcome (Figs 7b and 8b). Indeed, the importance of increased suprabasal labelling in stratified squamous epithelium was noted by Appleton *et al.* (1977) who emphasized the inevitably enhanced proliferative activity (larger growth fractions, shortened cell cycle times) consequent upon cell replication outside the basal layer.

At the present time it is unclear whether identification of lesions with poor clinical outcome would significantly alter treatment protocols or ultimate disease progress, but it is not an unreasonable hypothesis that early identification should rationalize therapeutic interventions, targeting high-risk patients and lesions, and marshalling systemic therapies at the earliest possible stages of clinical management.

In the absence of improved 5-year survival rates and the increased incidence of oral cancer, particularly in younger patients, further research is urgently needed to identify and determine the usefulness of predictive agents in individual patient management.

Figure 3. Photomicrograph of histone mRNA labelled dysplastic ventral tongue leukoplakia ($\times 22$).

Figure 4. Ki67 labelling indices. Overall LI, left panel; basal and suprabasal LIs, right panel.

Figure 5. Cyclin A labelling indices. Overall LI, left panel; basal and suprabasal LIs, right panel.

Figure 6. Histone mRNA labelling indices. Overall LI, left panel; basal and suprabasal LIs, right panel.

Figure 7. Ki67 labelling indices and clinical outcome for the 11 patients.

Figure 8. Cyclin A labelling indices and clinical outcome for the 11 patients.

Figure 9. Histone mRNA labelling indices and clinical outcome for the 11 patients.

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