Comparison of cell proliferation in the centre and advancing fronts of oral squamous cell carcinomas using Ki-67 index

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Abstract. The cell proliferation status of 60 oral squamous cell carcinomas from Sri Lankan subjects was examined by immunohistochemistry using the Ki-67 index. A comparison was made between the indices derived from the centre of the tumours and those derived from the invasive fronts of the same tumours. There was a positive correlation between the two indices suggesting a clonal expansion of malignant cells, but the mean index derived for the invasive fronts (29.75 ± 11.64) was significantly higher than the mean index for the body of these tumours (25.65 ± 11.64). Thus, at a given time, more peripheral cells at the invasive front are proliferating and this compartment is likely to be more informative in prognostic and other behavioural studies involving the cell cycle. In squamous carcinomas, increased and uncontrolled cell proliferation at the invasive front may be one feature contributory to the invasion.

INTRODUCTION

Rapid and uncontrolled growth of malignant neoplasms compared with normal tissue may be caused by an increased proliferation of cells, a smaller rate of cell loss, or both. Apoptosis is one of the key modes of cell loss. Even though both cell proliferation and cell loss by apoptosis are equally important in tumourigenesis, more attention has been paid to cell proliferation than cell loss. Numerous independent studies have indicated that the degree of cell proliferation is a potentially important tumour marker of considerable prognostic relevance for human cancers (Hall *et al.* 1988; Shepherd *et al.* 1988; Tubina & Courdi 1989; Kreipe *et al.* 1993). The immunohistochemical detection of Ki-67 nuclear protein as a method of assessment of cell proliferation is now routine in experimental pathology and has several advantages over other methods (Brown & Gatter 1990; Warnakulasuriya & Johnson 1996). A close association of Ki-67 protein expression with that of the proliferative compartment of epithelial tissues has been reported. However, the precise function of this protein is still unknown.

There have been comparatively few studies of the Ki-67 proliferation index in squamous cell carcinomas of the head and neck. Those reported, with two exceptions (one oral and one oesophageal) (Kuwano *et al.* 1998), have not systematically examined differences in cell proliferation in different compartments of a tumour. A considerable body of evidence now exists showing that the invasive front of a neoplasm contains morphological information of greater prognostic significance than elsewhere in the tumour (Bryne 1991). The morphological information included in Bryne's criteria include, degree of keratinization, cellular pleomorphism, mitotic activity, size of invading nests or islands and the host response. The objective of this study was to separately assess cell proliferation in the body and infiltrative fronts of oral squamous cell carcinomas (OSCC). The null hypothesis was that these indices would not vary.

MATERIALS AND METHODS

Tissue sampling

The pathological samples for this study were selected from the archives of two diagnostic histopathology laboratories in Sri Lanka located at the University Dental School, Peradeniya and the General Hospital, Kandy. Sixty formalin-fixed, paraffin-embedded OSCCs with clear evidence of invasive fronts at the deep surface of the biopsy were chosen. The mean age of the patients was 59.07 ± 11.87 and there were 48 males and 12 females in the study sample. By T staging, 16 tumours were T1, 36 were T2 and 8 cancers were at T3.

Six-micron-thick sections were cut and mounted on saline-coated slides. For ease of practical manipulation, slides were immunostained in three batches of 20. Phosphate-buffered saline (PBS) was used as the diluent for washing and rinsing steps throughout the immunohistochemistry protocol, except for the purposes of antigen retrieval.

Positive and negative controls

One section from each tumour block was used as the internal negative control by omitting the primary antibody and by incubating with the diluent buffer (PBS). An oral cancer sample known to show good Ki-67 labelling from a previous study acted as a positive control. One positive control was included for each immunohistochemical cohort.

Staining procedure

Slides were de-waxed, sections were re-hydrated and incubated in 0.5% hydrogen peroxide (H_2O_2) in 70% methanol in PBS for 20 min to eliminate endogenous peroxidase activity. After washing in PBS, the slides were heated to 100 °C in a laboratory microwave oven (Toshiba ER665ET, 650 W) at medium setting for a total of 10 min in two cycles of 5 min in 0.01 M sodium citrate buffer (pH 6.0) for antigen retrieval. The buffer level was checked between each cycle and adjusted if necessary. The slides were then bench cooled for 20 min and washed twice with gentle shaking in PBS twice for 5 min.

The sections were incubated with normal goat serum (1 : 30 dilution in PBS) for 20 min to block non-specific immune reactions. From each tumour sample, one section was incubated with the primary antibody for a period of 1 hour (monoclonal antibody Ki-67 (MIB-1; dilution 1 : 30, Immuno-tech, Cat No; 0505) and a second section was incubated with PBS for the same time interval as the negative control. The working dilution of the primary antibody was optimized after a series of trial experiments. Once primary incubation was completed, the slides were rinsed twice with PBS and the sections were then incubated with biotinylated secondary antibody (Daco-Duet) (dilution

1 : 100 in PBS) for 20 min. Following a further PBS wash, the sections were treated with biotin-streptavidin complex (Strept ABC, Dako-Duet) (dil 1 : 100) for a further 20 min. Visualization was with 0.05% diaminobenzidine tetra hydrochloride (DAB) chromagen (Sigma Cat No: 5637) in PBS in the presence of 0.1% hydrogen peroxide for 10 min. The sections were thoroughly rinsed in tap water, counter stained with Harris' haematoxylin, dehydrated in a graded series of ethanols (70, 90 and 100%) and cleared with xylene twice, 5 min each and mounted in DePex.

Assessment of immunohistochemically stained sections

Sections stained with Ki-67 antibody were examined under light microscopy. Ten samples, which did not react with the monoclonal antibody MIB-1, were excluded from the study. Fifty tumours, positive for Ki-67 proliferation antigen were assessed quantitatively. Cell counts were made at $\times 400$ magnification using a 10×10 squared eye piece graticule (Graticules Ltd) with a conventional light microscope.

The invasive fronts of each tumour were graded by scoring the five histopathological characteristics (see Introduction) reported by Bryne *et al.* (1989, 1991) using all available microscopical fields ($\times 25$ objective) from each tumour section. From the 50 tumours, 147 fields with the worst Bryne's score were selected for Ki-67 estimation. All tumour cell nuclei in the chosen microscopic fields were counted and the Ki-67 labelling index expressed as a proportion of labelled cells.

In the case of counting nuclei within the main bulk or centre of the tumour, three or more consecutive microscopic fields were selected. This was done by moving the slide first to position the graticule on the centre of the tumour, then moving it to the right by one microscope field. The count of tumour cell nuclei was made in three or more consecutive fields by moving the slide to the left, until over 1000 nuclei from the centre of the tumour were scored.

Mean Ki-67 index for each tumour was estimated as the percentage of immunoreactive nuclei among the total number of nuclei counted, separately for the body or the bulk of the tumour and for the selected invasive fronts.

Mitotic index

Cells in mitosis were identified using criteria earlier described for human oral epithelium (Warnakulasuriya 1976). Mitotic nuclei were counted separately in the tumour fronts and within the body/bulk of the tumours in the same microscopic fields ($\times 25$ objective) selected for Ki-67 counts. All tumour nuclei in the same fields were counted and the mitotic index was expressed as a percentage of total cells.

For both Ki-67 stained nuclei and mitotic cells, reproducibility was determined by counting and recounting replicates of the same fields until consistency was reached (data not shown). One observer performed all the counts to eliminate interobserver variability.

Statistical methods

The paired Student's *t*-test was used to evaluate the differences in the means of Ki-67 and mitotic indices. Any correlation of the established index at the two sites was performed with the Pearson correlation coefficient expressed as *r*.

RESULTS

Ki-67 positive nuclei were clearly and easily identified by their brown nuclear staining. Distinction between stained and unstained nuclei was unequivocal. Cytoplasmic staining did not

Table 1. Mean Ki-67 indices of 50 oral squamous cell carcinomas (for the centre/body and the invasive fronts)

Site	Counting	Mean Ki-67 index†	Mean mitotic index†
Invasive front of the tumour	All nuclei	29.75 ± 11.64	4.06 ± 0.39
Centre of the tumour	More than 1000 tumour nuclei	25.65 ± 11.52	3.23 ± 0.21

* $P < 0.05$, † $P < 0.05$.

Figure 1. Correlation of Ki-67 indices from the centre and the invading fronts from individual tumours.

occur accept in the mitotic phase. Mitotic figures stained darker brown than other positive cells and the cytoplasm showed yellowish brown staining. Variation in staining intensity between different tumours ranged from light to dark brown. No difference in gradient of intensity of staining between nuclei in the same tumour was observed. Clustering of positively stained nuclei was present alongside variably sized negative areas in several tumours which thus showed an heterogeneous pattern of reactive cell distribution. However, distribution of positive cells was homogeneous in other tumours. Basal and parabasal layers were positive for Ki-67 proliferation antigen in normal oral epithelium when present. The distribution of stained nuclei within tumour bodies varied from little to nil in central areas showing cellular maturation and keratinization, to high reactivity in the more actively proliferating peripheral areas (basal compartment) of tumour islands. Not all peripheral cells in the tumour body were positive. Conversely, most or all peripheral nuclei in the invading tumour islands were uniformly positive.

The mean Ki-67 index for the selected invasive fronts was 29.75 ± 11.64 and the mean Ki-67 index for the body/bulk of the tumour was 25.65 ± 11.64 (Table 1). The mean Ki-67 index for the invasive fronts was significantly higher than the mean Ki-67 for the bulk of the tumour ($P < 0.05$). The individual Ki-67 indices within a case also showed a significant correlation ($r = 0.78$; $P = 0.03$) between the body and invasive fronts of the tumours (Fig. 1).

Figure 2. Correlation of Ki-67 index (Ki) and Mitotic index (Mi) in the invading front (a) and body/centre of the tumour (b).

The mean mitotic index for the same invasive fronts was 4.06 ± 0.39 and the mean mitotic index for the body/bulk of the tumour was 3.23 ± 0.21 (Table 1). The mean mitotic index for the invasive fronts was significantly higher than the mean mitotic index for the bulk of the tumour ($P < 0.05$). The individual Ki-67 indices and mitotic indices within the invasive fronts of the tumours or the body showed significant correlation ($r = 0.41$; $r = 0.49$) (Fig. 2a and b).

Mean Ki-67 and mitotic indices by T stage of cancer is shown in Table 2. The number of cancers in T3 stage was small. No significant differences in levels of cell proliferation were noted by T stage.

Table 3 shows the distribution of the present 50 oral squamous cell carcinomas, in relation to the Ki-67 labelling index ranges employed in previously reported studies. At the invasive

Table 2. Mean Ki-67 and mitotic indices of 50 oral squamous cell carcinomas by tumour size

Clinical T stage	Mean Ki-67 index	Mean mitotic index
T1	25.1 $\pm$ 10.5	4.8 $\pm$ 4.5
T2	30.0 $\pm$ 11.43	3.6 $\pm$ 1.8
T3	37.2 $\pm$ 11.94	4.4 $\pm$ 1.5

No significant differences in the group means were found except in the Ki-67 index of groups T1 versus T3, $P = 0.045$.

Table 3. Distribution of 50 oral squamous cell carcinomas according to various Ki-67 LI ranges reported in the literature

	LI grading			LI grading			LI grading		
	< 10%	10–30%	> 30%	< 10%	10–50%	> 50%	< 20%	20–50%	> 50%
Invasive front	0	28 (56%)	22 (44%)	0	46 (92%)	4 (8%)	12 (24%)	34 (68%)	4 (8%)
Centre	0	37 (74%)	13 (26%)	0	48 (96%)	2 (4%)	18 (36%)	30 (60%)	2 (4%)

fronts less than 8% of tumours in the present sample showed a proliferative index of more than 50%. At the centre of the tumours only 4% showed this feature.

DISCUSSION

Fast-growing tumours have been shown to be deeply invasive and are known to have a poorer prognosis, compared with slowgrowing tumours (Tubiana & Courdi 1989). Experimental evidence suggests that the degree of cellular proliferation within a tumour holds promise to enable an estimate of their biological aggression (Sledge *et al.* 1988). Chauval *et al.* (1989) showed that patients with a tumour labelling index (LI) greater than 15.5% had a significantly poorer survival than patients with a lower tumour LI in head and neck SCCs. It has been also shown that the proportion of Ki-67 positive cells increases with histopathological grade in oral squamous cell carcinomas (Zoeller *et al.* 1993). Few reported studies have made a distinction in areas of the tumour selected for scoring. As the tumour invasive front is likely to be the most significant in terms of invasion, we examined the state of cell proliferation at invasive fronts and compared this with the index derived from the centres of the tumours.

The cell proliferation at invasive tumour fronts was greater than that of tumour bodies and this difference was statistically significant ($P < 0.05$). A similar result has previously been reported for oesophageal squamous cell carcinomas where the deep margin showed a higher proliferation index than central areas (Kuwano *et al.* 1998).

Accumulation of MIB-1 immunoreactive proliferating tumour cells at the invasive front of oral squamous cell carcinomas provides further supportive evidence for the putative biological significance of this particular area in epithelial malignancies (Bryne *et al.* 1992, 1995). Findings of previous studies, as well as the present results, show that the invasive front is the best prognostic area for growth fraction estimation in oral squamous cell carcinomas because of the highly proliferative activity of this zone compared with the rest of the tumour (Piffko *et al.* 1996).

Increased and uncontrolled cell proliferation at the invasive front may be one feature contributory to invasion. It would be worthwhile to systematically examine cell proliferation in oral verrucous carcinomas (which do not metastasize) to assess whether their peripheral cells have lower levels of proliferation and compare these with the invasive fronts of OSCC.

The results of the present study also showed that there is a significant correlation ($r = 0.78$) between the mean Ki-67 index in the invasive front and the body of the tumour in individual cases. This may suggest a clonal evolution of tumour nests with similar proliferative behaviour. However, the correlation of Ki-67 index and mitotic index of each tumour both at the tumour front and the body was low ($r = 0.41$; $r = 0.49$).

Recorded Ki-67 estimates, using Ki-67/MIB-1 antibodies from earlier studies were compared with the present study (Table 4). Of these 16 cell proliferation studies on head and neck cancers reported between 1990 and 2002, nine of them have been performed on OSCCs. Monoclonal antibody Ki-67 (MIB-1) has been used in over half of these studies and most authors have resorted to the use of formalin-fixed, paraffin-embedded biopsies. A mean labelling index for Ki-67, has been established only in seven of the 16 studies reported. Comparison of results proved difficult due to differences in data analyses, data presentation (including the use of mean or median indices), ranges and arbitrary gradings too low, medium or high. None examined the potential differences between morphological compartments of tumours.

Even though the immunohistochemical determination of Ki-67 proliferation antigen using monoclonal antibody MIB-1 is a straightforward method to assess proliferation in archival tissues, the literature reveals that it has not been used frequently in this type of study. When employing this method, caution must be exercised for a number of reasons. One of the pitfalls remains different degrees of tissue conservation and fixation resulting in heterogeneity in the pattern of staining, resulting in under staining or false negative staining. In the present study, 10/60 squamous cell carcinomas were unsuitable for Ki-67 detection. Absence of expression of Ki-67 nuclear protein has been observed in nutritionally deprived cells (Baisch & Gerdes 1987) and therefore special care should be taken to avoid the involvement of necrosed areas. Because of the heterogeneous nature of cell proliferation within any given tumour, it is important to screen the whole tumour and by use of sections at different levels of the same tumour to overcome the intratumour heterogeneity. In addition, the number of cells that need to be counted to obtain a representative index is not yet clearly defined. Counting over 500 tumour nuclei is generally accepted as a minimum requirement (Brown & Gatter 1990). The most important question to be answered is about sampling areas for counting immunoreactive cells. Most studies including the present one, have assumed that cells in all the phases of the cycle namely G_1 , S, G_2 and M are evaluated, by immunocytochemistry. However, it has been shown that cells which are in the early G_1 phase, could have been excluded from the estimate due to insufficient threshold of Ki-67 protein (Gerdes *et al.* 1984). Compared with the whole cell cycle, the early G_1 phase cells that may or may not have been included in the estimated mean value, would be proportionately very small. Alternatively, Scott *et al.* (1991) have shown that immunohistochemical measurement of Ki-67 may slightly overestimate the proliferative fraction of tumours.

Another important aspect to consider is that, by immunohistochemistry, we assess the fraction of proliferative cells or the state of cell proliferation and not the cell proliferation rate. A tumour with a slow cell cycle could have many cells in cycle with high mean Ki-67 index but still have a relatively slow proliferation rate. A tumour with a short cell cycle could be highly proliferative but could have few cycling cells resulting in a low mean Ki-67 index (Sarbia *et al.* 1996). It would be possible to overcome this problem with the introduction of double labelling of tissues *in vitro* (Warnakulasuriya 1976) but this requires fresh tissue and could not be undertaken on archival material. Pre-treatment *in vivo* labelling by intravenous administration of

Table 4. A summary of reported studies on Ki-67 labelling indices of oral and head and neck carcinomas

Site	Authors	Sample	Population	Fixation	Antibody	Ki-67 index (mean/median/range)
Oral	1. Kearsley <i>et al.</i> 1990	42	Australian	Frozen	Ki-67 (monoclonal)	2–52% (range)
	2. Girod <i>et al.</i> 1993	85	German	Formalin/paraffin	Ki-67-MIB-1	–
	3. Warnakulasuriya <i>et al.</i> 1994	20	British	Frozen	Ki-67 (monoclonal)	27.1
	4. Piffko <i>et al.</i> 1996	100	German	Formalin/paraffin	Ki-67-MIB-1	–
	5. Allison <i>et al.</i> 1998	24	British	Ethanol/paraffin	Ki-67 (monoclonal)	–
	6. Girod <i>et al.</i> 1998	103	German	Formalin/paraffin	Ki-67-MIB-1	14.6 (well) 16.6 (mod)
	7. Ng <i>et al.</i> 1999	56	Chinese	Formalin/paraffin	Ki-67-MIB-1	41.6 (poorly) # 2.24 (basal); 1.91 (supra basal)
	8. Thompson <i>et al.</i> 2002	4	British	Formalin/paraffin	Ki-67-MIB-1	27.2–39.5
	9. Tumuluri <i>et al.</i> 2002	47	Australia	Formalin-paraffin	Ki-67 (monoclonal)	1958 mm ²
	10. Dissanayake 2003*	50	Sri Lankan	Formalin/paraffin	Ki-67/MIB-1	29.48 (invasive front)
Head and neck	1. Jones <i>et al.</i> 1994	77	British	Frozen	Ki-67 (monoclonal)	25.65 (body)
	2. Roland <i>et al.</i> 1994	79	British	Frozen	Ki-67 (monoclonal)	29.8 (median)
	3. Slootweg <i>et al.</i> 1994	15	Netherlands	Formalin/paraffin	Ki-67-MIB-1	27.8 (median)
	4. Roland <i>et al.</i> 1996	12	German	Formalin/paraffin	Ki-67-MIB-1	–
	5. Silvestrini <i>et al.</i> 1997	42	Italy	Formalin/paraffin	Ki-67 (monoclonal)	19.0
	6. Couture <i>et al.</i> 2002	304	USA	Formalin/paraffin	Ki-67-MIB1	–
	7. Liu <i>et al.</i> 2003	80	USA	Formalin/paraffin	Ki-67 (monoclonal)	< 20% 41% tumours > 20% 59% tumours

*Present study.

bromodioxuridine (BrdUrd) allows determination of LI by flow cytometry, but this measurement has been shown, in a multicentre study, to be a weak predictor of prognosis (Begg *et al.* 1999).

Here, in oral squamous cell carcinomas, more cells from the invasive front compared with the main bulk or the centre of the tumours have been shown to be in the proliferative state. The differences are significant, refuting the null hypothesis. At any given time, more cells at the invasive front are proliferating, confirming that this part of the tumour is likely to be more informative in studies involving cell-cycle control and other prognostic indicators.

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