ORAL DISEASES

Oral Diseases (2010) 16, 68–75. doi:10.1111/j.1601-0825.2009.01611.x

www.wiley.com

ORIGINAL ARTICLE

Ki-67 expression in non-tumour epithelium adjacent to oral cancer as risk marker for multiple oral tumours

MA González-Moles[1], M Bravo[2], I Ruiz-Ávila[3], F Acebal[4], JA Gil-Montoya[1], S Brener[5], F Esteban[6]

[1]*Oral Medicine Department, School of Dentistry, University of Granada, Granada, Spain;* [2]*Preventive and Public Health in Dentistry Department, School of Dentistry, University of Granada, Granada, Spain;* [3]*Pathology Department, "Complejo Hospitalario", Jaen, Spain;* [4]*Oral and Maxillofacial Surgery Department, Neurotraumatological Hospital, "Complejo Hospitalario", Jaen, Spain;* [5]*Oral Medicine Department, School of Dentistry, University of Sao Paulo, Sao Paulo, Brazil;* [6] *Otorhinolaryngology Department, Virgen del Rocio University Hospital, Sevilla, Spain*

OBJECTIVE: The aim of this study was to determine whether the differential assessment of epithelial proliferation is useful to diagnose premalignant fields and assess the risk of multiple tumours.

MATERIAL AND METHODS: We analysed 83 oral carcinomas with associated non-tumour epithelium classified as distant or close according to its distance (>or<1 cm) from the invasion point, and as squamous hyperplasia, mild, moderate, severe dysplasia or carcinoma *in situ*. Twenty-five healthy oral mucosa samples were used as controls. An immunohistochemical technique was applied using Mib-1. Ki-67 in premalignant epithelium was assessed in basal layer, parabasal layer, medium and upper third.

RESULTS: Parabasal expression was significantly higher or showed a tendency to be higher in close and distant epithelia with any histological grade than in the controls. Parabasal Ki-67 significantly differed between distant epithelia associated with multiple *vs* single tumours ($P < 0.001$) and between distant epithelia associated with multiple tumours *vs* controls ($P < 0.001$). This difference was not observed between distant epithelia associated with single tumours and controls ($P = 0.175$). The cut-off point that differentiated epithelia associated with multiple tumours was >50% of Ki-67 + parabasal cells in distant epithelia, which yielded 0.88 sensitivity and 0.79 specificity.

CONCLUSIONS: The concept of a precancerous field may be linked to an increase in the proliferative activity of parabasal cells.

Oral Diseases (2010) **16**, 68–75

Keywords: second tumour; premalignant field; proliferation; Ki-67; oral cancer

Correspondence: MA González-Moles, Facultad de Odontología; Paseo de Cartuja s/n, 18071 Granada. Spain. Tel: +34 958243804, Fax: +34 9582409080, E-mail: magonzal@ugr.es
Received 27 March 2009; revised 4 June 2009; accepted 26 June 2009

Introduction

The development of multiple tumours is a major issue for the prognosis of oral squamous cell carcinoma (OSCC) patients after local surgery and radiotherapy, with an incidence of 17–30% and annual risk of 3–10% (Tabor *et al*, 2001). When the index tumour has been completely resected, a new cancer might be attributable to a genetically altered field (Braakhuis *et al*, 2003). Second tumours that appear on precancerous fields are designated second field tumours because they share early oncogenic alterations with the first tumour and with the field, mainly mutations of TP53 and loss of heterozygosity (LOH) in 3p, 9p and 17p. By contrast, second primary tumours are genetically independent, recurrences are genetically identical and localized in the same anatomical area and metastases are genetically identical and localized in different organs (Mao *et al*, 1996; Partridge *et al*, 2000; Rosin *et al*, 2000). The diagnosis of premalignant fields is important because of the increased risk of developing multiple field tumours (Braakhuis *et al*, 2002) and its implications for prevention, therapy and prognosis. Currently, a reliable diagnosis of premalignant fields requires the use of molecular techniques (mutational and LOH analysis) (Braakhuis *et al*, 2002) that are not routinely applied because of their cost and complexity. Early genetic alterations of chromosomal loci 3p, 9p and 17p in these fields imply a function loss of important tumour suppressor genes and the acquisition of proliferative and expansive advantages by field cells, which presumably substitute healthy oral mucosa and create a mucosal area with an enhanced risk of developing multiple tumours (Tabor *et al*, 2003). Tabor *et al* (2003) found an excellent correlation between oncogenic changes in premalignant fields (LOH in 3p, 9p, 17p, 8p, 13q and 18q) and the percentage of proliferative cells in the whole thickness of dysplastic epithelia. However, premalignant fields do not necessarily show recognizable clinical (leucoplasia and erythroplasia) or histopathological

(epithelial dysplasia) features. Furthermore, only some cell layers (basal and parabasal) are normally proliferative in non-dysplastic epithelia. Proliferative cell counts in the whole epithelial thickness do not allow evaluation of the implications and the clinical and prognostic relevance of cell proliferation at different epithelial levels (Gonzalez-Moles *et al*, 2000). Given the essentially proliferative nature of premalignant fields, the objective of this study was to determine whether the differential assessment of epithelial proliferation by layer, applying immunohistochemical methods (Ki-67 expression), is a useful tool to diagnose premalignant fields and assess the risk of developing multiple tumours.

Patients and methods

We studied 83 OSCC patients who had non-tumour epithelium associated with the invasive tumour. They were derived from 67 patients aged 27–91 years (60.0 ± 11.6) under treatment at the Jaen Hospital Complex (Spain); 51 patients (76.1%) were male and 38 (73%) were smokers or ex-smokers. After the study had been approved by the hospital ethics committee, the hospital records of patients were reviewed and data were gathered on the clinicopathological characteristics of lesions. The histological grade of non-tumour epithelium adjacent to the invasive carcinoma was assessed on 4-μm tissue sections. Non-tumour epithelium adjacent to the carcinoma was classified as distant or close to the tumour according to its localization at a distance of more or <1 cm from the point of invasion. The distance was calculated by counting 1000 cells in the basal layer from this point and considering the average cell diameter to be 10 μm (van Houten *et al*, 2002). We adopted 1 cm as the cut-off point because this is half the distance (2 cm) proposed by Hong *et al* (1990) to distinguish a second tumour from a recurrence, hence cell proliferation at a greater distance may be considered secondary to the expansive growth of a premalignant field. In addition, the UK guidelines (Woolgar, 2006) has established 0.5 cm as the safe distance for considering a surgical margin to be tumour-free, therefore we believe that any tumour arising at a greater distance than 1 cm should not be deemed a recurrence of the primary tumour. Although we acknowledge that this decision is to some degree arbitrary and open to debate, there are no data in the literature to allow us to differentiate in a more precise and relevant manner between epithelia that are proximal or distant from a tumour. WHO classifications (Barnes *et al*, 2005) were used to diagnose epithelial dysplasia and assess the epithelial histological grade as squamous hyperplasia, mild dysplasia, moderate dysplasia, severe dysplasia or carcinoma *in situ*. Clinicopathological variables were considered missing when not found in the clinical records or, in the case of non-tumour epithelium histological grade, when there was inadequate non-tumour epithelium for a reliable evaluation of dysplasia.

The presence of second tumours was recorded according to the following criteria: derivation from surface epithelium and not from the deep surgical margin of the index tumour (Johnson *et al*, 2005); complete resection of the primary tumour according to conventional histopathological assessment of the surgical margin (Tabor *et al*, 2002); presence of at least 2 cm of non-tumour epithelium between second and index tumours, based on clinical, surgical and histopathological findings (Hong *et al*, 1990); and onset ≥6 months after appearance of the index tumour (metachronic carcinoma; Hashibe *et al*, 2005).

The control group comprised 25 samples of healthy oral epithelium adjacent to benign non-inflammatory mucosal lesions (mucoceles, 22 cases) obtained from the paraffin block archives of the Pathology Department of the Jaen Hospital Complex and gingival mucosa adjacent to premolars that were extracted for orthodontic purposes in adults (three cases) obtained from the school of Dentistry of Granada. Inclusion criteria for controls were: clinical and histological mucosa normality, absence of inflammatory infiltrate and derivation from a non-smoking patient (data from hospital clinical records). The case and control groups were from patients within the same age range.

Immunohistochemistry

For the immunohistochemical staining, 4-μm sections were cut from paraffin blocks. The peroxidase–antiperoxidase technique was used, performing immunohistochemical analysis by means of the avidin–biotin method. The immunohistochemical study was performed automatically, using Autostainer Link equipment (Dako, Carpintería, CA, USA) and EmVision™ *FLEX* reagents (K8002; Dako, Carpintería, CA, USA). The manufacturer's instructions were rigorously followed. This system allows deparaffinization and rehydration followed by recovery of the heat-induced epitope. A reproducible recovery of the epitope is ensured by completely loading the slide recipient, guaranteeing an identical heating of all sections in each cycle. We used the primary antibody Mib-1 (Dako), recommended by the manufacturer for this automatic system. Counterstaining was performed using the EmVision™ *FLEX* Hematoxylin system (K8008; Dako), which gives a light blue nuclear stain, followed by permanent mounting of the samples in DPX. For the negative control, the primary antibody was replaced with phosphate buffer saline. For the positive control, tissue was used from an OSCC known to intensively express Ki-67. The result was considered positive when a brown colour appeared in cell nuclei. Ki-67 expression in premalignant epithelium was assessed in four randomized high-power fields (40×), dividing the epithelium thickness into four compartments: basal layer, parabasal layer (formed by approximately four cell rows), medium third and upper third. Total cell number and number of positive cells were counted in each field and compartment, obtaining a mean expression percentage in each epithelial compartment for every case.

Cases and controls were assigned to one of the following categories: 0% positive cells (−), 1–25% positive cells (+), 26–50% positive cells (++), 51–75% positive cells (+++), or >75% positive cells (++++)

(Figure 1). The histological and immunohistochemical analyses were always performed by the same examiner (MAGM), who was blind to the clinical stage, treatment or course of the disease.

Statistical methods

SPSS-Windows v.15.0 (SPSS Inc., Chicago, IL, USA) was used for descriptive purposes. *P*-values were calculated using SUDAAN v.7.0 (Research Triangle Institute, Durham, NC, USA), with design WR (with-replacement) to account for clustering (multiple oral cancers within patients). The procedures used are indicated in Table 4 and 5 footnotes. In some analyses the original Ki-67 expression scale was converted into a quantitative scale by considering the mean value of each interval, as follows: 0% (−) for < 1% interval; 13% (+) for 1–25%; 38% (++) for 26–50%; 63% (+++) for 51–75% and 88% (++++) for 76–100% interval. The discriminant ability of parabasal Ki-67 expression in distant epithelium to differentiate between patients with multiple and single tumours was analysed as follows. First, the proportions of multiple tumours observed for each Ki-67 expression value were contrasted by means of the Mantel-Haenszel test for linear association, based on a chi-square distribution (χ^2_{MH}) with 1 d.f. Then, the area under the Receiver Operating Characteristic (ROC) curve (and standard error) was calculated by means of the Wilcoxon statistic (Hanley and McNeil, 1982). Finally, sensitivity (Se) and specificity (Sp) were calculated for parabasal Ki-67 expression in distant epithelium at the cut-off point giving the best (the highest) Youden Index, i.e. Se + Sp − 1 (Youden, 1952).

Results

Table 1 shows results for tumour clinicopathological variables. The most frequent localization was the tongue (45 cases; 54.2%) and the most frequent clinical presentation was ulceration of malignant appearance (33 cases; 46.5%). In this series, 25 cases (32.5%) were T1, 29 cases (37.7%) T2, 49 cases (63.6%) N0 and 61 cases (79.2%) M0. Table 2 shows data on the multiple tumours in 10 of the patients (15%). Among the 65 non-tumour epithelia close to invasive carcinomas (close epithelia), 16 (24.6%) had squamous hyperplasia, 14 (21.5%) mild dysplasia, 25 (38.5%) moderate dysplasia and 10 (15.4%) severe dysplasia/carcinoma *in situ*. Among the 48 epithelia distant from invasive carcinomas (distant epithelia), 28 (58.3%) had squamous hyperplasia, five (10.4%) mild dysplasia, 14 (29.2%) moderate dysplasia and one (2.1%) severe dysplasia/carcinoma *in situ*. The examiner did not report on the histological grade of adjacent epithelia in 18 of the 83 samples because of inadequate tissue availability. Only 48 of the 83 tumours in the study had non-tumour epithelium distant from the invasive carcinoma. Table 3 shows the percentage expression of Ki-67 in control epithelia and in non-tumour epithelia adjacent to carcinomas. The maximum level of parabasal Ki-67 expression (++++) was found in 49.2% of close epithelia and in 47.9% of distant epithelia, while no or very low parabasal Ki-67 expression was detected in 18.4% of close epithelia. Ki-67 expression was compared between control epithelia and non-tumour epithelia adjacent to the carcinoma as a function of the epithelial histological grade (Table 4). Parabasal expression was significantly higher or showed a tendency to be higher in close and distant epithelia with any histological grade than that in the controls. Cell proliferation in basal and parabasal layers and medium third of close epithelia was significantly higher in dysplasias *vs* hyperplasias. Ki-67 expression in parabasal layers and medium third of close epithelia was significantly higher in severe dysplasias/carcinomas *in situ* than in other epithelial histological grades. In distant epithelia, only the expression of Ki-67 in the medium-third differentiated dysplasias from

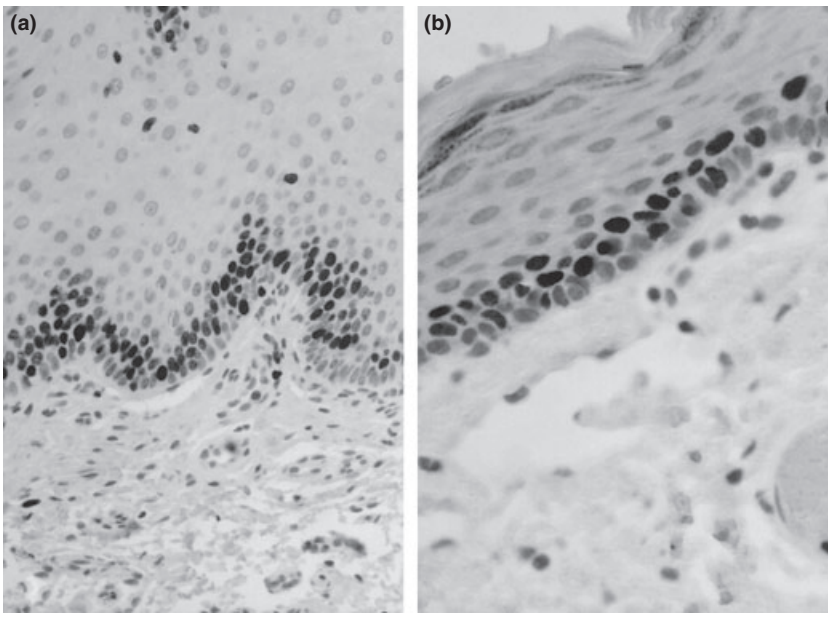

Figure 1 (**a**) Ki-67 expression in parabasal layers of distant epithelium from a patient who developed second tumours. Sixty-three per cent (+++) of the parabasal cells are proliferating. Very few basal cells express Ki-67 (immunohistochemical technique, 20×; scale bar = 100 μm). (**b**) Ki-67 expression in parabasal layers in distant epithelium of a patient developing a single tumour. A total of 37% of the parabasal cells are proliferating (immunohistochemical technique, 40×; scale bar = 100 μm)

Table 1 clinicopathological parameters of tumours ($n = 83$)[a]

Variable	n *(%)*
Localization	
Tongue	45 (54.2)
Mouth floor	13 (15.7)
Floor + tongue	10 (12.0)
Trigone	4 (4.8)
Other[b]	11 (3.3)
Clinical presentation	
Ulcer	33 (46.5)
Tumour	22 (31.0)
Leucoplakia + tumour	8 (11.3)
Other[c]	8 (11.3)
Missing	12
Size	
T1	25 (32.5)
T2	29 (37.7)
T3	10 (13.0)
T4	13 (16.9)
Missing	6
Adenopathies	
N0	49 (63.6)
N1	19 (24.7)
N2a	5 (6.5)
N2b	2 (2.6)
N2c	1 (1.3)
N3	0 (0.0)
NX	1 (1.3)
Missing	6
Metastasis	
M0	61 (79.2)
M1	1 (1.3)
MX	15 (19.5)
Missing	6
Stage	
I	20 (26.0)
II	19 (24.7)
III	19 (24.7)
IVa	18 (23.4)
IVb	0 (0.0)
IVc	1 (1.3)
Missing	6
Degree of differentiation	
Well differentiated	31 (41.9)
Moderately differentiated	26 (35.1)
Poorly differentiated	17 (23.0)
Missing	9

[a]Corresponding to 67 patients.
[b]Buccal mucosa + trigone ($n = 1$), buccal mucosa ($n = 3$), soft palate + buccal mucosa ($n = 2$), lower lip ($n = 2$) and gingiva ($n = 3$).
[c]Lichen planus + ulcer ($n = 2$), erythroleucoplakia ($n = 3$) and leucoplakia + ulcer ($n = 3$).

hyperplasias. Table 5 shows Ki-67 expression in control epithelia and those adjacent to oral tumours according to the presence or not of multiple tumours. We highlight the significant difference in parabasal expression of Ki-67 between distant epithelia associated with multiple *vs* single tumours and control ($P < 0.001$, respectively). This difference was not observed between distant epithelia associated with single tumours and the controls ($P = 0.175$). With regard to the discriminant ability of parabasal Ki-67 in distant epithelium to differentiate between patients with multiple *vs* single tumours, a linear association [χ^2_{MH} (1 gl) = 18.63, $P < 0.001$] was found, with an area under the ROC curve (±s.e.) of 0.83 ± 0.06 [95% confidence interval (CI): 0.69–0.92]. The optimal cut-off point (according to Youden criteria) was ++/+++ (>50% of Ki–67 + expression), which yielded 0.88 sensitivity (95% CI:0.68–0.97) and 0.79 specificity (95% CI:0.58–0.93).

Discussion

The main findings of this study were significantly higher cell proliferation rate in parabasal layers of non-tumour epithelia distant from multiple carcinomas than in epithelia distant from single carcinomas or control epithelia and the similar rate between epithelia distant from single carcinomas and the controls. Given the close association reported between Ki-67 expression and LOH in 3p, 9p and 17p (early oncogenic events in premalignant fields), with a sensitivity of 86% and specificity of 100% (Tabor *et al*, 2003), increased parabasal proliferation in epithelia distant from multiple carcinomas might identify them as premalignant fields. Accordingly, the second tumours in the present patients would appear to correspond to second field tumours. By contrast, the absence of significant differences in parabasal proliferation rate between epithelia distant from single tumours and controls ($P = 0.226$) presumably indicates that they did not arise from expansive premalignant fields. However, it was recently suggested that malignant epithelial clones may result not only from genetically altered somatic SC but also from oncogenic events that initially affect a parabasal cell (Gat *et al*, 1998; Zhu and Watt, 1999; Perez-Losada and Balmain, 2003; Costea *et al*, 2006). In accordance with this hypothesis, our results indicate that parabasal cells may be targets for oncogenic events that increase their cell proliferation rate but maintain their capacity to develop terminal differentiation. This would augment the pool of cells susceptible to new oncogenic events and hence the risk of multiple tumour development. Our results point to a further step in oral oncogenesis that involves the loss of terminal differentiation in some parabasal cells, as the proliferation rate in more superficial areas of epithelium was significantly or close-to-significantly higher in dysplasias *vs* hyperplasias and significantly higher in severe dysplasias/carcinomas *in situ vs* other histological grades. The idea that precancerous fields are associated with greater proliferation in the parabasal layer also has implications for the growth of the fields. According to the current model of precancerous field expansion, the entire thickness of healthy epithelium would be replaced by genetically altered field cells (Braakhuis *et al*, 2003; Perez-Losada and Balmain, 2003), whereas our results indicate that only parabasal cells are replaced by parabasal cells with a higher proliferation rate. We therefore hypothesise that this advance border of only three or four cell layers (laminar expansion) would facilitate field growth.

Our statistical analysis showed that the parabasal proliferative cut-off point differentiating precancerous fields that developed multiple carcinomas from those that developed single carcinomas was >50%

Table 2 Clinicopathological characteristics of patients with multiple tumours ($n = 10$ patients and 27 tumours)

Patient	*Age*[a]	*Sex*	*Tumour*	*Localization*	*T*	*N*	*M*	*G. diferent.*
1	54	M	1	R mobile tongue	T1	N1	Mx	WD
			2	R base of tongue	T1	N1	Mx	WD
2	45	M	1	R tongue + floor	T4	N1	M0	MD
			2	L tongue + floor	T4	N1	M0	MD
3	61	M	1	R mouth floor	T4	N0	M0	WD
			2	L mouth floor	T4	N0	M0	MD
4	49	M	1	L base of tongue	T1	N1	Mx	MD
			2	L mobile tongue	T1	N1	Mx	MD
5	62	F	1	L mobile tongue	T2	N0	M0	MD
			2	Anterior mouth floor	T2	N0	M0	MD
6	70	F	1	L base of tongue	T2	N0	M0	PD
			2	L mobile tongue	T2	N0	M0	MD
			3	R mobile tongue	T2	N0	M0	MD
7	68	M	1	L mobile tongue	T2	N1	M0	MD
			2	L base of tongue	T2	N1	M0	MD
			3	Anterior mouth floor	T2	N1	M0	MD
8	52	F	1	L mobile tongue	T4	N0	M0	WD
			2	R base of tongue	T4	N0	M0	WD
			3	R oral mucosa	T3	N0	M0	WD
9	49	M	1	L anterior mouth floor	T1	N0	M0	MD
			2	R anterior mouth floor	T1	N0	M0	MD
			3	R base of tongue	T1	N0	M0	WD
10	63	F	1	Anterior lower gingiva	T3	N0	M0	MD
			2	R lower gingiva	T2	N0	M0	WD
			3	Lower lip	T2	N0	M0	WD
			4	R trigone	T2	N0	M0	WD
			5	L upper gingiva	T1	N0	M0	WD

[a]Mean ± s.d. = 57.3 ± 8.6 years.
R, right; L, left; WD, well differentiated; MD, moderately differentiated, PD, poorly differentiated.

Ki-67 + parabasal cells. Prospective studies are needed to verify the usefulness of this cut-off point to select patients with a risk of developing multiple tumours and to diagnose premalignant fields, for example, taking multiple intra-operative biopsies of oral mucosa at surgery of the primary tumour.

The presence of parabasal cells in proliferative states and their different significance as a function of their proliferation rate raise questions about the selection of controls in oral epithelium proliferation studies. Authors such as Tabor *et al* (2003) consider parabasal proliferation without proliferation in the medium and

Table 3 Percentage expression of Ki-67 in control epithelia ($n = 25$) and epithelia adjacent to oral tumours ($n = 83$)[a]

	Ki-67 expression (%)					
Epithelium	*Negative (0%)*	*+ (1–25%)*	*++ (26–50%)*	*+++ (51–75%)*	*++++ (76–100%)*	*Mean ± s.d.*[b]
Controls ($n = 25$)						
Basal layer	4	68	24	4	0	20.5 ± 14.5
Parabasal layer	0	56	44	0	0	24.0 ± 12.7
Medium third	100	0	0	0	0	0.0 ± 0.0
Upper third	100	0	0	0	0	0.0 ± 0.0
Adjacent epithelium close to tumour ($n = 65$)[c]						
Basal layer	0	33.8	23.1	16.9	26.2	46.8 ± 30.1
Parabasal layer	1.5	16.9	12.3	20	49.2	62.8 ± 29.7
Medium third	40	33.8	13.8	9.2	3.1	18.2 ± 23.2
Upper third	84.6	10.8	0	3.1	1.5	4.7 ± 15.5
Adjacent epithelium distant from tumour ($n = 48$)[d]						
Basal layer	0	81.3	4.2	4.2	10.4	23.9 ± 24.7
Parabasal layer	4.2	35.4	6.3	6.3	47.9	53.1 ± 36.3
Medium third	58.3	22.9	14.6	4.2	0	11.2 ± 17.2
Upper third	95.8	0	2.1	2.1	0	2.1 ± 10.5

[a]Corresponding to 67 patients.
[b]Mean ± standard deviation of percentage of expressing cells.
[c]The difference with the total 83 tumours corresponds to inadequate tissue availability.
[d]Only 48 of the 83 tumours in the study had non-tumour epithelium distant from the invasive carcinoma.

Table 4 Comparison of Ki-67 expression (mean ± s.d.)[a] between control epithelia ($n = 25$) and epithelia adjacent to oral tumours ($n = 83$)[b] as a function of histological grade

	Histological grade in adjacent epithelia					*Selected contrasts,* P-*value*[d]						
Epithelium	*Control (C)*	*Hyperplasia (H)*	*Mild dysplasia (L)*	*Moderate dysplasia (M)*	*Severe dysplasia/ carcinoma in situ* (S)	*Global* P-*value*[c]	*C vs H*	*C vs L*	*C vs M*	*C vs S*	*H vs L + M + S*	*H + L + M vs S*
Close epithelium (*n*)[e]	25	16	14	25	10							
Basal layer	20.5 ± 14.5	19.2 ± 17.1	61.2 ± 26.8	54.0 ± 30.5	53.0 ± 24.2	< 0.001	0.808	< 0.001	< 0.001	< 0.001	< 0.001	0.39
Parabasal layer	24.0 ± 12.7	45.0 ± 37.1	75.5 ± 19.0	62.0 ± 29.3	75.5 ± 13.2	< 0.001	0.027	< 0.001	< 0.001	< 0.001	0.017	0.026
Medium third	0.0 ± 0.0	0.0 ± 0.0	11.1 ± 9.8	22.4 ± 20.3	46.7 ± 31.3	< 0.001	g	< 0.001	< 0.001	< 0.001	< 0.001	0.002
Upper third	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	2.6 ± 5.3	24.0 ± 33.8	0.058	g	g	0.013	0.056	0.057	0.069
Distant epithelium (*n*)[f]		28	5	14	1							
Basal layer		15.7 ± 10.4	18.0 ± 11.2	39.8 ± 37.3	63.0 ± 0.0	0.263	0.167	0.642	0.303	h	0.163	h
Parabasal layer		44.2 ± 36.3	53.0 ± 37.9	68.4 ± 32.8	88.0 ± 0.0	< 0.001	0.021	0.063	< 0.001	h	0.074	h
Medium third		3.7 ± 8.4	12.8 ± 15.5	21.8 ± 20.0	63.0 ± 0.0	< 0.001	0.016	0.043	0.004	h	0.004	h
Upper third		1.4 ± 7.2	0.0 ± 0.0	0.0 ± 0.0	63.0 ± 0.0	0.348	0.302	g	g	h	0.602	h

[a]Mean ± standard deviation of percentage of expressing cells.
[b]Corresponding to 67 patients.
[c]SUDAAN REGRESS procedure to correct for clustering (multiple oral cancers within patients). For distant epithelia, the severe and moderate dysplasia categories were collapsed.
[d]SUDAAN DESCRIPT procedure to correct for clustering (multiple oral cancers within patients).
[e]In the case of epithelia adjacent to tumours, the difference with the total of 83 tumours corresponds to inadequate tissue availability.
[f]Only 48 of the 83 tumours in the study had non-tumour epithelium distant from the invasive carcinoma.
[g]Comparison using SUDAAN was not possible because all values were 0 in one category.
[h]Comparison was not possible due to lack of data in the severe dysplasia/CIS group.

Table 5 Comparison between Ki-67 expression (mean ± s.d.)[a] in control epithelium ($n = 25$) and epithelium adjacent to oral tumours according to the presence of multiple tumours ($n = 83$)[b]

	Multiple tumours			P-*value*			
Epithelium	*Control (C)*	*No (N)*	*Yes (Y)*	*Global*[c]	*C vs N*[d]	*C vs Y*[d]	*N vs Y*[d]
Close epithelium (*n*)[e]	25	42[e]	23[e]				
Basal layer	20.5 ± 14.5	37.4 ± 26.2	64.1 ± 29.7	<0.001	<0.001	<0.001	0.001
Parabasal layer	24.0 ± 12.7	52.3 ± 29.3	82.0 ± 19.3	<0.001	<0.001	<0.001	<0.001
Medium third	0.0 ± 0.0	14.2 ± 20.1	25.4 ± 27.0	<0.001	<0.001	<0.001	0.113
Upper third	0.0 ± 0.0	3.0 ± 10.4	7.7 ± 22.0	0.219	0.058	0.227	0.478
Distant epithelium (*n*)[f]		24	24				
Basal layer		17.2 ± 14.1	30.7 ± 30.8	0.451	0.412	0.438	0.305
Parabasal layer		33.3 ± 31.5	72.9 ± 29.8	<0.001	0.175	<0.001	<0.001
Medium third		5.3 ± 13.4	17.0 ± 18.9	0.003	0.05	0.003	0.06
Upper third		2.6 ± 12.9	1.6 ± 7.8	0.315	0.312	0.312	0.73

[a]Mean ± standard deviation of percentage of expressing cells.
[b]Corresponding to 67 patients.
[c]SUDAAN REGRESS procedure to correct for clustering (multiple oral cancers within patients).
[d]SUDAAN DESCRIPT procedure to correct for clustering (multiple oral cancers within patients). Note the absence of clustering for the C *vs*. N comparison.
[e]In the case of epithelia adjacent to tumours, the difference with the total of 83 tumours corresponds to inadequate tissue availability.
[f]Only 48 of the 83 tumours in the study had non-tumour epithelium distant from the invasive carcinoma.

upper third of the epithelium to be normal. In fact, studies rarely provide details on the origin of oral mucosa used as control, which is usually reported simply as healthy oral mucosa (Macluskey *et al*, 2000; Fan *et al*, 2006; Takeda *et al*, 2006; Abbas *et al*, 2007). We have the impression that the control samples used by many authors are mucosa obtained from edentulous areas obtained during implant placement or from apparently healthy areas distant from oral carcinomas and obtained during tumour surgery. We believe that these samples are not valid as controls, because edentulous areas subjected to masticatory trauma habitually develop frictional keratosis, presumably associated with an increase in the proliferation rate of parabasal cells. We do not believe that mucosa distant from oral carcinoma should be accepted as a control, as it may be undergoing proliferative precancerous changes that are not clinically or histopathologically evident. Finally, oral mucosa from a smoker should not serve as a control because tobacco may also generate proliferative changes. We consider the selection of controls to be a critical issue in this research line. Besides excluding the types of sample mentioned above, controls should be clinically and histopathologically normal and contain no inflammatory infiltrate. This approach is essential to establish the true value of epithelial and especially parabasal proliferation as a marker of a precancerous field.

Finally, this study detected epithelia close to the invasive carcinoma that had no or very low Ki-67 expression in all epithelial layers. In our opinion, this finding indicates that tumour development may not only be attributed to a progressive and slow accumulation of early and late oncogenic events. It may also result from the sudden development by a cell of oncogenic alterations that allow it to acquire proliferative and invasive phenotypes that are not dependent on pre-existing disorders in a precancerous field.

In short, according to the present findings, an increase in the proliferation rate in parabasal layers of oral epithelia distant from an OSCC is an indicator of the risk of developing new tumours. Further studies are required to evaluate the genetic changes associated with an increase in parabasal proliferation and to determine their similarity with observations in premalignant fields.

Author contributions

Gonzalez-Moles has participated in all stages of the study and manuscript preparation. Bravo contribution has been the study design and the data analysis. The rest of the author has contributed in the literature research, data acquisition and manuscript editing and review.

References

Abbas NF, Labib El-Sharkawy S, Abbas EA, Abdel Monem El-Shaer M (2007). Immunohistochemical study of p53 and angiogenesis in benign and preneoplastic oral lesions and oral squamous cell carcinoma. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* **103:** 385–390.

Barnes L, Eveson JW, Reichart PA, Sidransky D (2005). *World health organization classification of tumours. Pathology and genetics. Head and neck tumours*. World Health Organization: Lyon.

Braakhuis BJM, Tabor MP, Leemans CR, Van Der Waal I, Snow GB, Brakenhoff RH (2002). Second primary tumors and field cancerization in oral and oropharyngeal cancer: molecular techniques provide new insights and definitions. *Head Neck* **24:** 198–206.

Braakhuis BJ, Tabor MP, Kummer JA, Leemans CR, Brakenhoff RH (2003). A genetic explanation of Slaughter's concept of field cancerization: evidence and clinical implications. *Cancer Res* **63:** 1727–1730.

Costea DE, Tsinkalovsky O, Vintermyr OK, Johannessen AC, Mackenzie IC (2006). Cancer stem cells – new and potentially important targets for the therapy of oral squamous cell carcinoma. *Oral Dis* **12:** 443–454.

Fan GK, Chen J, Ping F, Geng Y (2006). Immunohistochemical analysis of P57 (kip2), p53 and hsp60 expressions in premalignant and malignant oral tissues. *Oral Oncol* **42:** 147–153.

Gat U, DasGupta R, Degenstein L, Fuchs E (1998). *De novo* hair follicle morphogenesis and hair tumors in mice expressing a truncated beta-catenin in skin. *Cell* **95:** 605–614.

Gonzalez-Moles MA, Ruiz-Avila I, Rodriguez-Archilla A, Martinez-Lara I (2000). Suprabasal expression of Ki-67 antigen as a marker for the presence and severity of oral epithelial dysplasia. *Head Neck* **22:** 658–661.

Hanley JA, McNeil BJ (1982). The meaning and use of the area under a receiver operating characteristic (ROC) curve. *Radiology* **143:** 29.

Hashibe M, Ritz B, Le AD, Li G, Sankaranarayanan R, Zhang ZF (2005). Radiotherapy for oral cancer as a risk factor for second primary cancers. *Cancer Lett* **220:** 185–195.

Hong WK, Lippman SM, Itri LM *et al* (1990). Prevention of second primary tumors with isotretinoin in squamous-cell carcinoma of the head and neck. *N Engl J Med* **1990:** 323.

van Houten VMM, Tabor MP, van den Brekel MWM *et al* (2002). Mutated P53 as molecular marker for the diagnosis of head and neck cancer. *J Pathol* **198:** 476–486.

Johnson N, Franceschi S, Ferlay J *et al*. (2005) Squamous cell carcinoma. In: Barnes L, Eveson JW, Reichart P, Sidransky D, eds. *Pathology and genetics. Head and neck tumours*. Lyon: IARC Press, pp. 168–175.

Macluskey M, Chandrachud LM, Pazouki S *et al* (2000). Apoptosis, proliferation, and angiogenesis in oral tissues. Possible relevance to tumour progression. *J Pathol* **191:** 368–375.

Mao L, Lee JS, Fan YH *et al* (1996). Frequent microsatellite alterations at chromosomes 9p21 and 3p14 in oral premalignant lesions and their value in cancer risk assessment. *Nat Med* **2:** 682–685.

Partridge M, Pateromichelakis S, Phillips E, Emilion GG, A'Hern RP, Langdon JD (2000). A case-control study confirms that microsatellite assay can identify patients at risk of developing oral squamous cell carcinoma within a field of cancerization. *Cancer Res* **60:** 3893–3898.

Perez-Losada J, Balmain A (2003). Stem-cell hierarchy in skin cancer. *Nat Rev Cancer* **3:** 434–443.

Rosin MP, Cheng X, Poh C *et al* (2000). Use of allelic loss to predict malignant risk for low-grade oral epithelial dysplasia. *Clin Cancer Res* **6:** 357–362.

Tabor MP, Brakenhoff RH, van Houten VM *et al* (2001). Persistence of genetically altered fields in head and neck cancer patients: biological and clinical implications. *Clin Cancer Res* **7:** 1523–1532.

Tabor MP, Brakenhoff RH, Ruijter-Schippers HJ *et al* (2002). Multiple head and neck tumors frequently originate from a single preneoplastic lesion. *Am J Pathol* **161:** 1051–1060.

Tabor MP, Braakhuis BJM, van der Wal JE *et al* (2003). Comparative molecular and histological grading of epithelial dysplasia of the oral cavity and the oropharynx. *J Pathol* **199:** 354–360.

Takeda T, Sugihara K, Hirayama Y, Hirano M, Tanuma JI, Semba I (2006). Immunohistological evaluation of Ki-67, p63, CK19 and p53 expression in oral epithelial dysplasias. *J Oral Pathol Med* **35:** 369–375.

Woolgar JA (2006). Histopathological prognosticators in oral and oropharyngeal squamous cell carcinoma. *Oral Oncol* **42:** 229–239.

Youden WJ (1952). Index for rating diagnostic tests. *Cancer* **3:** 32.

Zhu AJ, Watt FM (1999). Beta-cathenin signalling modulates proliferative potential of human epidermal keratinocytes independently of intercellular adhesion. *Development* **126:** 2285–2298.