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Original

Analysis of cell proliferation and pattern of invasion in oral squamous cell carcinoma

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Abstract: Cell proliferation markers play an important role in the biological behavior of neoplasms. This study investigated the immunohistochemical expression of PCNA, Ki-67 and Cyclin B1 proteins based on the pattern of cell invasion in oral squamous cell carcinoma (OSCC). A total of 39 OSCC specimens and 13 samples of normal oral mucosa (control) were immunohistochemically analyzed. Protein expression was evaluated according to World Health Organization – Histological Malignancy Grading (WHO-HMG) and a specific grading system for invasion, graded from 1 to 4, varying from a consistently well-defined "pushing" border to diffuse infiltration and cellular dissociation, and was then correlated with clinical features. We found higher expression of Ki-67 and Cyclin B1 in OSCC when compared with the control group. High Ki-67 expression levels were more commonly seen in the floor of the mouth than in the tongue ($P = 0.009$). Cyclin B1 showed a positive correlation with histological grade, according to WHO-HMG criteria ($P = 0.01$). Our results suggest that Cyclin B1 is a reliable proliferation marker for indicating degree of tumor proliferation. Correlations between PCNA, Ki-67, Cyclin B1 and invasive tumor front with overall survival were not observed. Further studies are needed in order to elucidate whether cell proliferation activity at the tumor invasion front is related to prognosis. (J Oral Sci 52, 417-424, 2010)

Keywords: PCNA; Ki-67; Cyclin B1; pattern of invasion; oral squamous cell carcinoma.

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Introduction

Several studies have shown that clinical and histopathologic factors have prognostic significance in patients with oral squamous cell carcinoma (OSCC). In 1973, Jakobsson et al. (1) proposed the grading of multiple histological parameters, but this notion never won wide acceptance. Bryne et al. (2) published an approach to histopathological malignancy grading in OSCC, exclusively evaluating the most advanced invasive tumor cell layers at the tumor-host interface. Several studies have since investigated the prognostic significance of invasive tumor front (ITF) grading in OSCC.

In order to classify tumors histologically, the World Health Organization-Histological Malignancy Grading (3), a system in which numerous morphological parameters are considered (e.g., number of mitosis, degree of keratinization, leukocytic infiltration), is used. In 1999, Spiro et al. (4) introduced a grading system for patterns of invasion (Invasive Tumor Front classification: ITFc).

Grades went from a consistently well-defined "pushing" border (Grade 1) to diffuse infiltration and cellular dissociation (Grade 4), based on the criteria used by Anneroth et al. (5) and Bryne et al. (2). They found that Grade 3 and 4 patterns at the tumor-host interface were associated with an increased incidence of nodal and distant metastases, as well as a significant decrease in survival rates (4). However, their study was based on oral tongue cancer and other studies would be necessary to confirm these data.

Tumor cell proliferation activity provides insights into tumor biology. Methods for assessing the state of cellular proliferation, such as flow cytometric S-phase fraction and mitotic frequency analysis, are impractical for routine diagnosis. The immunohistochemical assessment of cell proliferation has advantages over other techniques because the tissue architecture is intact and proliferating cells can be visualized and correlated to other histological features (6).

Various antigens, such as PCNA and Ki-67, have been used in the immunohistochemical analysis of cell proliferation. PCNA is a 36-kDa non-histonic nuclear polypeptide associated with the cell cycle, and is considered necessary for DNA replication and cell proliferation (7-9). The Ki-67 and Cyclin B1 markers are considered to be good indicators of the proliferative activity of a cell population (10,11). Ki-67 is a non-histonic nuclear protein that is present throughout all the active phases of the cell cycle (G1, S, G2, and M), but is absent from resting cells (G0), and reaches its peak concentration in phases G2 and M (12).

As the cell enters mitosis, phosphorylation of key components of subcellular structures causes the complete reorganization of cellular architecture. This phosphorylation is primarily due to the activation of Cyclin B1/cdc2 complexes (13). Cyclin B1 is a cytoplasmic protein that, when combined with another protein known as Cdk 1, forms the MPF (M-phase Promoting Factor). Cyclin B1 begins to accumulate during S and G2, reaching peak concentrations during mitosis, particularly in metaphase (14,15). The important function of these proteins in regulating cell cycle progression in normal cells means that elevated levels of Cyclin B1 proteins in oral carcinomas are likely to play a role in the increased proliferation of malignant cells.

The aim of this study was to investigate the expression of PCNA, Ki-67 and Cyclin B1 in tumor invasion front areas of OSCC by evaluating the clinical pathological implications of the results and correlating them with tumor invasion patterns, according to the criteria of the WHO-HMG (3) and the classification used by Spiro et al. (4).

Materials and Methods

Patients

Thirty-nine surgically excised specimens of OSCC were obtained from the archives of the Anatomopathology and Cytopathology Division of Araújo Jorge Hospital, Association of Cancer Combat of Goiás (Goiânia, Brazil), between 1996 and 2000. All patients were subjected to surgical treatment, and none received radiotherapy, chemotherapy or other modalities before surgery. Cases with areas of extensive necrosis or non-preserved morphological structures (observed microscopically) were excluded. Clinical data (gender, age, ethnicity, tobacco and alcohol consumption, tumor location, extension and TNM stage) and follow-up information (recurrence and death) were obtained from medical records. Thirteen specimens of normal oral mucosa obtained through esthetic surgical procedures or gingival tissue of patients underwent biopsies during a tooth extraction procedure acted as the control group. This study was approved by the institutional ethics committee for human subjects (protocol CEPMHA/HC/UFG 040/04).

Light microscopy

All specimens were fixed in 10% formalin buffer (pH 7.4), and were embedded in paraffin. The microscopic features were evaluated by analyzing one 5-μm section from each sample, stained routinely with hematoxylin and eosin (H&E). The obtained slides were analyzed by four calibrated oral pathologists using a digital image system projector (Olympus-BX41, OLY-200, Olympus America Inc., Center Valley, PA, USA). The ITF classification (ITFc) consisted of grading margin areas of tumors in four groups, as proposed by Spiro et al. (4): 1, consistently well-defined "pushing" border; 2, advancing edge of tumor infiltrating in solid cords, bands, or strands; 3, margins with small groups or cords of infiltrating cells; and 4, margins with marked cellular dissociation in small groups or even single cells (Fig. 1).

Immunohistochemistry

We used mouse anti-human PCNA monoclonal antibody (clone PC10, Dako, Copenhagen, Denmark; dilution: 1:2,000), anti-human Ki-67 (clone MM1, Novocastra, Newcastle, UK; dilution: 1:100) and anti-human Cyclin B1 (clone 7A9, Novocastra, Newcastle, UK; dilution: 1:40). Briefly, paraffin-embedded tissues were sectioned (3 μm) and collected in serial sections on glass slides coated with 2% 3-aminopropyltriethylsilane (Sigma Chemicals, St. Louis, MO). Sections were deparaffinized by immersion in xylene, followed by immersion in alcohol and incubation with 3% hydrogen peroxide diluted in

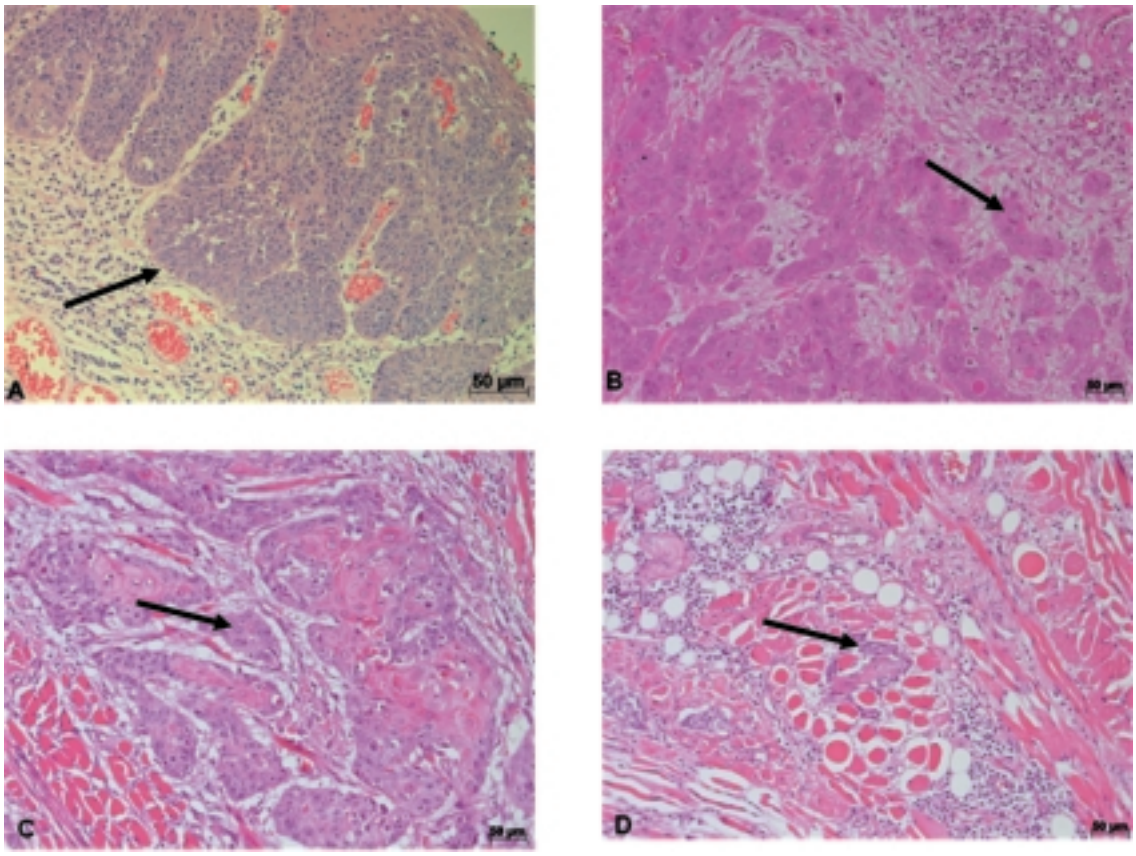

Fig. 1 Pattern of cell invasion in oral squamous cell carcinoma: (A) Grade 1: Tumors had well-defined "pushing" borders. (B) Grade 2: lesions, the advancing edge of the tumor infiltrated in solid cords, bands, or strands. (C) Grade 3: tumors had margins that contained small groups of infiltrating cells. (D) Grade 4: the host/tumor interface showed marked cellular dissociation in small groups or single cells. See arrows. (H&E, original magnification: ×200).

Tris-buffered saline (TBS; pH 7.4) for 40 min. Next, sections were immersed in citrate buffer (pH 6.0) for 20 min at 95°C for antigen retrieval. Soon afterwards, sections were blocked by incubation with 3% normal goat serum diluted in distilled water at room temperature for 20 min. Slides were then incubated with primary antibodies at 4°C overnight in a humidified chamber. After washing in TBS, sections were treated with labeled streptavidin-biotin (LSAB) kits (K0492, Dako). Sections were then incubated with 3,3'-diaminobenzidine (DAB) in a chromogen solution (K3468, Dako) for 2 to 5 min at room temperature. Finally, sections were stained with Mayer's hematoxylin and covered. Negative controls were obtained by omitting primary antibodies, which were substituted with 1% PBS-BSA and non-immune mouse serum (X0910, Dako).

Quantitative and qualitative analysis

A total of 611 images of the 39 cases were captured using a digital system (Olympus-BX41, OLY-200, Olympus America Inc.). Immunohistochemical staining was recorded using quantitative grading, taking into account the number of ITF cells that showed an unequivocal positive reaction in relation to the total number of the cells present in an image. The results were evaluated by defining a threshold of positive staining for all sections before automated processing. Briefly, the threshold for positive signals was defined for each nuclear (Ki-67 and PCNA) and cytoplasmic (Cyclin B1) antibody. Color signals above the threshold were deemed to be positive, whereas signals below the threshold were regarded as negative. This process was performed using Image-Pro Plus (IPP) 4.0 (Media Cybernetics, Silver Spring, MA, USA). All images analyzed with IPP 4.0 were subsequently confirmed by a pathologist. The results are expressed as a percentage index (PI) based on average values from five fields in the ITF of OSCC, and from two fields for the control group.

Cut-off points for PI were mainly based on the median, but also took into consideration the frequency distribution

for each marker in order to avoid the formation of very small subgroups. PCNA was categorized considering values ≤44.8% to be low and values >44.8% to be high expression. Ki-67 was categorized considering values ≤22% to be low and values >22% to be high expression, whereas for Cyclin B1, values ≤15.1% were considered to be low and values >15.1 were considered to be high expression.

Statistical analysis

Analyses were performed using the SPSS statistical package, version 11.0 (SPSS Inc., Chicago, IL, USA). Pearson's test was used to assess the associations between different numerical variables. Comparisons for continuous variables not following the normal distribution were performed with two groups using the Mann-Whitney test. Survival analyses were performed using the product-limit procedure (Kaplan-Meier method) and differences between categories were estimated by the log-rank test, with the date of the histological diagnosis as the starting point. Significance was set at 0.05.

Results

In this study, there were eight female patients (20.5%) and 31 male patients (79.5%), ranging in age from 33 to 90 years (mean 60.7 years). Thirty-seven patients (94.9%) smoked and 28 (71.7%) consumed alcohol. The most common tumor site was the tongue (13 cases: 33.3%), followed by the floor of the mouth (11 cases: 28.2%), while 15 cases had other tumor sites (gingiva, palato, buccal mucosa). Of the lesions, 31 (79.5%) were ulcer-infiltrative and eight (20.5%) were ulcero-vegetant. There was no recurrence in 31(79.5%) of 39 cases, and local metastases was seen in 29 (74.4%) cases. Survival rates varied from 4 months to 8 years. These results are summarized in Table 1.

PCNA and Ki-67 immunohistochemical staining was predominantly nuclear, while Cyclin B1 was cytoplasmatic, in both the normal oral mucosa and OSCC groups (Fig. 2). PCNA expression varied between 1.1 and 100% (mean, 49.1%); for Ki-67, it was 7.1-55% (mean, 25.2%), and for Cyclin B1, it was 2.2-63.9% (mean, 17.6%). According to the WHO-HMG (3), four samples (10.3%) were well differentiated, 31 (79.5%) were moderately differentiated and four (10.3%) were poorly differentiated. According to ITFc, four (10%) cases were Grade 1; 12 (30.8%) were Grade 2; 15 (38.5%) were Grade 3; and eight (20.5%) were Grade 4.

Table 1 Patient demographic and clinical characteristics (n = 39)

		OSCC group	
		n	%
Age	≥ 60 yrs	22	56.4
	< 60 yrs	17	43.6
Gender	Male	31	79.5
	Female	8	20.5
Location	Tongue	13	33.3
	Floor of the mouth	11	28.2
	Other	15	38.5
T stage	T1	3	7.7
	T2	11	28.2
	T3	10	25.6
	T4	14	35.8
	T0	1	2.7
WHO-HMG	1	4	10.3
	2	31	79.4
	3	4	10.3
ITFc	Grade 1	4	10.2
	Grade 2	12	30.7
	Grade 3	15	38.4
	Grade 4	8	20.7
Survival	≥ 24 months	26	66.6
	< 24 months	13	33.4

T: Stage – TNM system,
WHO-HMG: World Health Organization – Histological Malignancy Grading
ITFc: Invasive Tumor Front classification

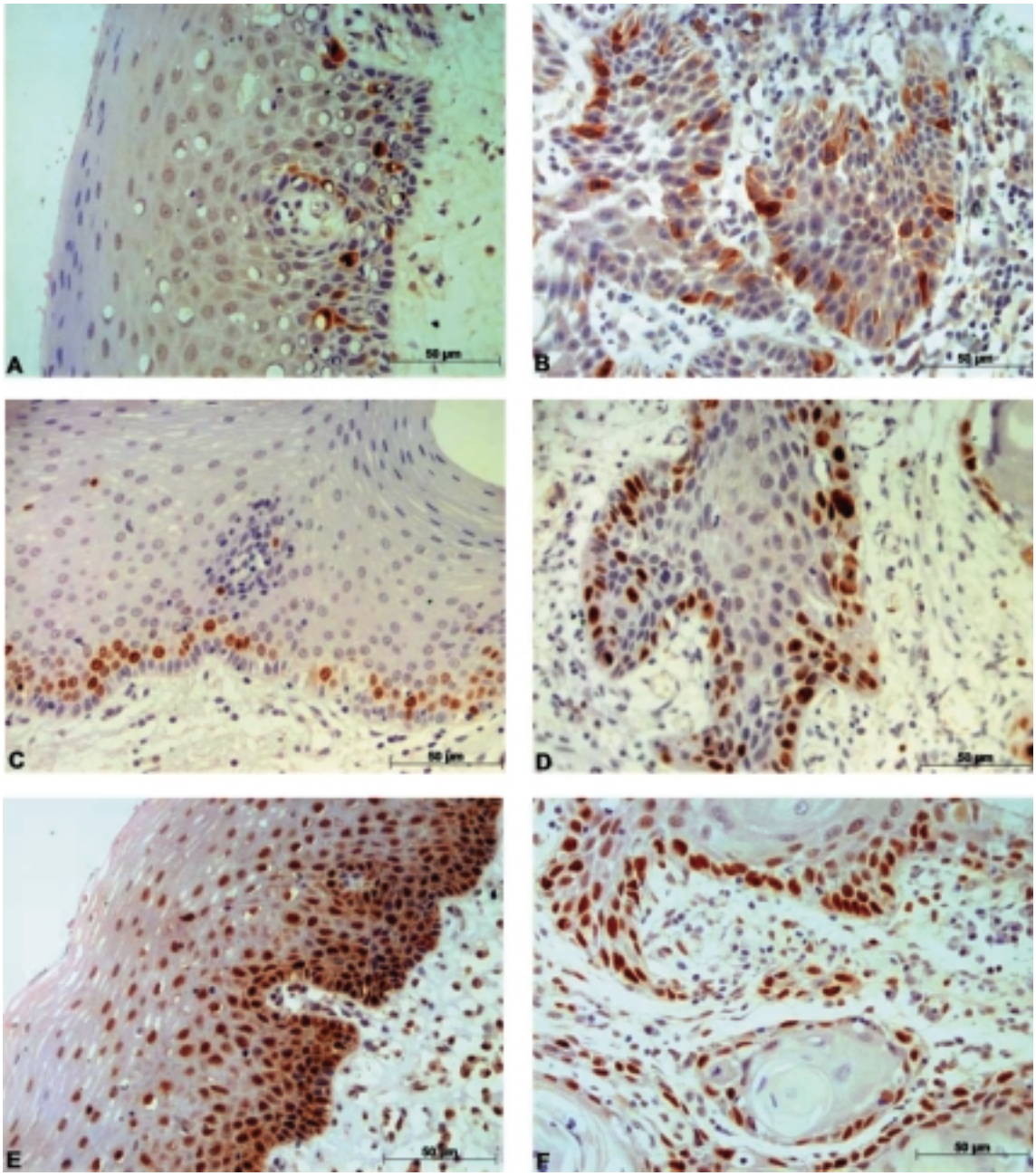

Fig. 2 Immunohistochemical staining patterns of Cyclin B1, Ki-67 and PCNA in normal oral mucosa and invasive tumor front of OSCC. Normal stratified squamous epithelium with Cyclin B1 staining in basal and parabasal layers (A) and high expression of this protein in tumor tissue (B) (cytoplasmatic staining). Nuclear expression of Ki-67 in normal epithelium (C) staining basal and parabasal layers and important expression in proliferating cells at the invasive tumor front (D). Prominent nuclear staining of normal epithelial tissue (E) and uniform staining; however, this staining was lower in tumor cells (F). (original magnification: ×400)

PCNA, Ki-67 and Cyclin B1 expression in OSCC and normal oral mucosa:

Ki-67 and Cyclin B1 showed higher expression levels in the OSCC group than in the control group ($P < 0.05$). On the other hand, PCNA expression was higher in the control group than in the OSCC group (Table 2).

PCNA, Ki-67 and Cyclin B1 proteins:

There were no statistically significant relationships between proliferative markers in the ITF of OSCC with regard to clinical pathological features such as ethnicity,

Table 2 Comparisons of PCNA, Ki-67 and Cyclin B1 expression between the control and OSCC groups (Mann-Whitney test)

		Cases	Mean-rank	*P*-value
PCNA	Control	13	43.0	< 0.001
	OSCC	39	20.9	
Ki-67	Control	13	18.0	< 0.001
	OSCC	39	29.3	
Cyclin B1	Control	13	9.0	< 0.001
	OSCC	39	32.3	

Table 3 Associations between Cyclin B1, PCNA and Ki-67 expression, and age (Spearman's test)

		Cyclin B1	PCNA	Ki-67	Age
Cyclin B1	r-value	1.00	0.06	0.35	0.10
	P-value	-	0.72	0.03	0.55
PCNA	r-value	0.06	1.00	-0.15	0.25
	P-value	0.72	-	0.36	0.13
Ki-67	r-value	0.35	-0.15	1.00	0.13
	P-value	0.03	0.36	-	0.42
Age	r-value	0.10	0.25	0.13	1.00
	P-value	0.55	0.13	0.42	-

Table 4 Correlations between PCNA, Ki-67 and Cyclin B1 expression, ITFc and WHO-HMG, and overall survival after five years (Kaplan-Meier test)

Variable		Cases	Overall-survival (%)	log-rank (*P*)
PCNA	expression ≤ 44.8%	20	92	0.14
	expression > 44.8%	19	76	
Ki-67	expression ≤ 22%	20	82	0.65
	expression > 22%	19	85	
Cyclin B1	expression ≤ 15.1%	20	90	0.60
	expression > 15.1%	19	73	
ITFc	1 and 2	16	88	0.82
	3 and 4	23	78	
WHO	I	4	100	0.52
	II	31	85	
	III	4	50	

smoking, alcohol consumption, TNM stage, metastasis and HMG according to Spiro et al. (4). High PCNA expression was correlated with female gender ($P = 0.012$) and the floor of the mouth ($P = 0.02$). High Ki-67 expression was correlated with the floor of the mouth, as compared with the tongue ($P = 0.009$). On the other hand, the Ki-67 expression was not correlated with other clinical pathological features. High Cyclin B1 levels of expression correlated with higher WHO-HMG (3) of the tumors ($P < 0.05$) but not with other clinical pathological features.

Association between proteins

A weak association between Ki-67 and Cyclin B1 ($r = 0.35$; $P = 0.03$) was observed, but associations between other proteins or age groups were not seen (Table 3).

Survival analyses

There were no correlations between PCNA, Ki-67, Cyclin B1, WHO-HMG and ITFc, and overall patient survival in this study (Table 4).

Discussion

Proliferation is considered to be a fundamental biological process because of the role it plays in the growth and maintenance of tissue homeostasis. Particularly in OSCC, proliferation has traditionally received much attention. Investigations related to proliferation assessment have become common in histopathology as a means of predicting the behavior of tumors, such as the likelihood of local recurrence, metastatic potential, and the growth of metastases, as well as disease-free survival and survival until death. Various methods are now available to assess certain properties of cellular proliferation and are readily applicable to daily histopathological practice, such as the evaluation of molecular proliferation markers such as PCNA, Ki-67 and Cyclin B1.

In addition, the involvement of resection margins and the pattern of tumor invasion have also been reported to be important when predicting local recurrence and survival in surgically treated patients (4). In this study, we retrospectively assessed the significance of PCNA, Ki-67 and Cyclin B1 in ITF areas as prognostic factors according to WHO-HMG (3) and the criteria of Spiro et al. (4).

Significant proliferative activity represented by Cyclin B1 and Ki-67 expression was observed in the OSCC group when compared with the control group. In fact, this has also been observed in previous studies (16-20). On the other hand, the presence of PCNA-positive cells outside the basal layer (in the G0 phase) was probably one reason for the higher PCNA expression in the control group than in the OSCC, as observed in this study (6). In fact, not all studies have confirmed the correlation between PCNA expression and proliferation, mitotic activity and Ki-67 proliferative labeling index (21). This is probably because PCNA is also involved in DNA repair. As there is DNA repair is active and ongoing in many tumors, PCNA may also be upregulated in non-proliferating cells. Indeed, in some tumors, 100% of cells show positive staining (6). Therefore, we conclude that PCNA cannot be considered a reliable proliferation marker in tumors.

Our results showed that high Ki-67 expression correlated more closely with the floor of the mouth, as compared with the tongue. Therefore, the more aggressive behavior of carcinoma in the floor of the mouth appears to be correlated with a higher proliferation activity, as indicated by the Ki-67 expression. On the other hand, Ki-67 expression was not correlated with other clinical pathological features. Previous studies reported no significant correlation between histological grade and Ki-67 expresion (19,20).

The classification used by Spiro et al. (4) in this research was not correlated with the tumoral proliferation index in ITF. In their study, they found that tumor patterns of Grades 3 or 4 at the tumor-host interface were associated with aggressive behavior in tongue cancer. However, when considering the WHO-HMG classification (3), a positive correlation with Cyclin B1 expression seems to reflect differences in the proliferative aspects of biological behavior. Tumor differentiation is thus apparently correlated with Cyclin B1 expression. Similarly, a previous report showed that less differentiated tumors presented higher levels Cyclin B1 expression (16). Therefore, we believe that Cyclin B1 is a reliable proliferation marker for indicating degrees of tumor malignancy.

A weak association between Cyclin B1 and Ki-67 was observed, and this suggests an irregularity in cell cycle progression during the G2/M phases, as observed in a previous study (16).

Correlations between proliferative markers and tumor size (T) were not observed in this study. This finding agrees with the report by Tumuluri et al. (22), who also investigated the relationships among proliferating cells at the ITF.

With regard to overall survival, the expression of proliferative markers independent of WHO-HMG and/or ITFc was not signifcant. However, higher expression of Cyclin B1 tended to be associated with a lower rate of survival. Indeed, morphological differences between the different patterns of invasion may not be implicated in actual tumor proliferative activity. It is also important to note that tumor proliferation activity analysis, as represented by PCNA, Ki-67 and Cyclin B1 expression in this study, excludes factors that have a major impact on patient survival, such as the presence of metastases and systemic conditions.

In conclusion, PCNA, Ki-67 and Cyclin B1 expression at the ITF is correlated with some clinical pathological features (location of the tumor, gender). Moreover, Cyclin B1 showed a positive correlation with the histological grade, when WHO-HMG criteria was used. Therefore, the use of Cyclin B1 and Ki-67 markers to characterize the interface tumor-front of invasion may lead to better understanding of the behavior of OSCC.

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