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Immunohistochemical detection of Ki-67 is not associated with tumor-infiltrating macrophages and cyclooxygenase-2 in oral squamous cell carcinoma

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BACKGROUND: An inflammatory component consisting of cells and chemical mediators may influence the proliferation and dissemination of the oral squamous cell carcinoma (OSCC). In the present study, we evaluated the possible relationship between Ki-67, tumor-associated macrophages (TAMs), and COX-2 in OSCCs. In addition, the immunodetection of these proteins was associated with different histological grades of malignancy, including invasive and *in situ* tumors.

METHODS: Twenty-seven OSCC cases were examined by light microscopy using criteria adopted WHO, and immunohistochemistry for Ki-67, CD68, and COX-2 using EnVision System in invasive and *in situ* lesions. Immunohistochemical detection of these proteins was assessed and scored for COX-2, and results were compared with their histological grades of malignancy.

RESULTS: A correlation between Ki-67, COX-2, and CD68 was not found. Histological grade of malignancy (HDM) was associated with the Ki-67 immunostaining ($P = 0.00$), but this was not observed regarding both CD68 ($P = 0.51$) and COX-2 ($P = 0.89$). Furthermore, there was a COX-2 overexpression in 62.96% of the sample, and a high density of TAMs in both OSCCs and *in situ* carcinomas.

CONCLUSIONS: Imunolabeling for Ki-67 was directly correlated with less-differentiated tumors, suggesting that this marker may contribute to understand the biological behavior of OSCC, and help to distinguish risk groups of OSCC. Furthermore, the lack of correlation between Ki-67, COX-2, and CD68 indicates that the latter two markers may play a pivotal role in oral carcinogenesis. However, further studies are needed to clarify their contribution for cell proliferation and tumor differentiation.

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Introduction

The development of oral squamous cell carcinomas (OSCCs) involves a microenvironment in which an inflammatory component consisting of cells and chemical mediators favors the proliferation and dissemination of the tumor (1) Macrophages play a key role because they represent the major cellular component of the tumor stroma, acting as regulators of tumor growth (2) and serving as an independent prognostic factor in tumors such as hepatocellular carcinomas (3).

Ki-67 is a large non-histone protein of approximately 395 kDa, which has been used as a marker of proliferative activity during the G1, S, G2, and M phases of the cell cycle (4, 5). In view of its high sensitivity and specificity in the characterization of cell proliferation in neoplastic tissues, Ki-67 has been widely used as a reliable marker in various studies on oral neoplasms (6, 7).

Tumor-associated macrophages (TAMs) have been the target of attention because they are poorly related to the local destruction of tumor cells and rather exert functions in favor of the tumor, such as the expression of cytokines and mitogenic and proangiogenic enzymes (8), suppression of adaptive immunity (9, 10), induction of metalloproteinase (MMP) expression (11), and secretion of cyclooxygenase-2 (COX-2) (12).

Cyclooxygenase-2, which is produced by macrophages or by the tumor cells themselves, is generally induced only in response to proinflammatory factors (13) and is considered to be an oncoprotein because it is expressed in diverse solid malignant tumors, including renal

tumors (14), tumors of the gastrointestinal tract (15), and oral neoplasms (16). The key events in tumorigenesis related to COX-2 involve the stimulation of mitogenesis (17) and angiogenesis (18), a decrease in E-cadherin expression (19), resistance to apoptosis (20), an increase in invasive potential (21), and mediation of immunosuppression (22).

Little is known about the participation of macrophages, COX-2, and Ki-67 in OSCCs. We therefore investigated, using immunohistochemistry, the possible relationship between these proteins in OSCCs. In addition, the immunodetection of these proteins was associated with different histological grades of malignancy, including invasive and *in situ* tumors.

Materials and methods

After approval by the Ethics Committee, 27 cases diagnosed as OSCC that included *in situ* and invasive tumors were selected. The anatomopathological records and clinical charts were obtained from the archives of the Surgical Pathology Service of the School of Dentistry, Federal University of Bahia (FO-UFBA), and of the School of Dentistry, Federal University of Rio de Janeiro (Department of Pathology and Oral Diagnosis, FO-UFRJ). As the samples were incisional biopsies performed for initial diagnosis, none of the patients had undergone chemo- or radiotherapy.

For morphological analysis, the formalin-fixed and paraffin-embedded material was cut into 4-μm-thick sections. After staining with hematoxylin–eosin, the material was reviewed by an experienced pathologist (J.N.S) to classify the cases based on the grade of differentiation of the tumor according to the World Health Organization (WHO) classification (23).

For immunohistochemistry, the formalin-fixed and paraffin-embedded material was cut into 3-μm-thick sections. The tissue sections were deparaffinized, and for antigen retrieval, conditions included sections boiled in citrate buffer solution, pH 6.0 for 40 min in a water bath at 95–97°C for CD68 and Ki-67. COX-2 followed the same conditions in a pressure cooker for 3 min. The sections were immersed in methanol with 3% hydrogen peroxide for 20 min to quench endogenous peroxidase activity. Afterwards, monoclonal antibodies against Ki-67 (clone MIB-1, Dako, A/S, Copenhagen, Denmark; dilution 1:50), CD68 (clone KP1, Dako, A/S; dilution 1:100), and COX-2 (Cayman Chemical, Ann Arbor, MI, USA; dilution 1:200) were applied overnight using the EnVision™ System (Dako Corporation, Carpinteria, CA, USA). Immunohistochemical reactions were developed with 3′3-diaminobenzidine (Dako Corporation) as chromogenic peroxidase substrate, and the slides were counterstained with Harris hematoxylin. Tissue fragments of normal oral mucosa served as positive control. An additional positive control consisted of a fragment of transplanted kidney with known presence of inflammation. Negative control included replacement of the primary antibody with non-immune bovine serum albumin.

For the analysis of immunostaining, the slides were examined by a single previously trained observer (D.S.VB) using a light microscope at a final magnification of 400 × . Ki-67 immunoexpression was analyzed taking 1000 epithelial cells as a reference for the analysis of each case. The proliferative index of the tumor was expressed as the percentage of immunostained nuclei in relation to the total number of nuclei counted. For the analysis of macrophage density, CD68-positive cells were defined as macrophages. TAMs were analyzed as described by Ueno et al. (24), quantifying positively stained cells by visualization of the five most confluent (hot spots) fields. Next, the mean of the three highest counts obtained for the five fields was calculated. Macrophage density was reported per mm^2 tissue. COX-2 expression in epithelial tumor cells was analyzed according to the method by Chan et al. (25). A total of 1000 cells in representative fields were taken as a reference, and the intensity and proportion of staining were analyzed. For each section, staining intensity was evaluated based on the predominant intensity, attributing a score of 1 to 4, where 1 = no staining, 2 = weak and diffuse cytoplasmic staining, 3 = moderate granular cytoplasmic staining, and 4 = strong granular cytoplasmic staining. The proportion of staining was defined as the percentage of labeled cells (0–100%). Multiplication of intensity (1–4) by proportion (0–100%) resulted in a score ranging from 0 to 400. COX-2 immunoreactivity was then established as follows: 0 = product of 0 to 10 (negligible staining), 1 = 11 to 99 (weak staining), 2 = 100 to 199 (moderate staining), and 3 = 200 to 400 (strong staining). Stroma was only analyzed descriptively.

Statistical comparisons were performed by ANOVA and chi-square test. Pearson's correlation test was used to establish the correlation between proteins. A P value < 0.05 was considered statistically significant.

Results

Histopathological aspects

Histological grading according to the WHO classification showed that eight (29.62%) of the tumors were ''well differentiated'', nine (33.33%) were ''moderately differentiated'', and six (22.22%) were ''poorly differentiated''. Four (14.81%) tumors presented histological characteristics of an *in situ* carcinoma.

The expression of Ki-67

All tumors exhibited Ki-67-positive nuclei (Fig. 1A,B), with the expression of this protein being detected in 29.68% of *in situ* tumors nuclei, 32.25% of well-differentiated OSCCs nuclei, 38.29% of moderately differentiated carcinomas nuclei, and 33.93% of poorly differentiated carcinomas nuclei. Expression of Ki-67 was higher in moderately and poorly differentiated OSCCs compared with *in situ* carcinomas and well-differentiated tumors ($P = 0.00$). Similarly, moderately differentiated tumors presented a higher rate of Ki-67-

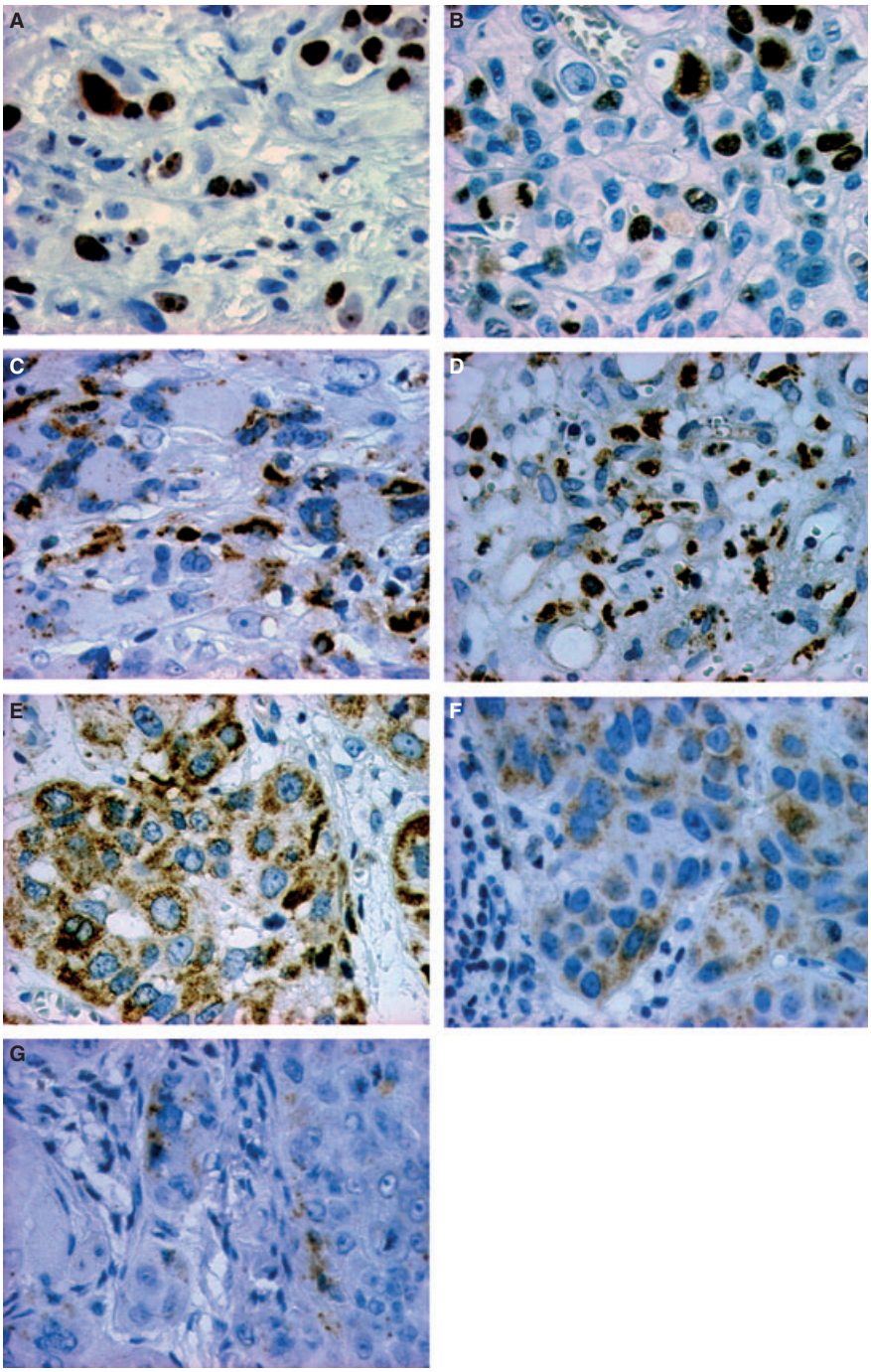

Figure 1 Immunohistochemical staining of oral squamous cell carcinoma. (A, B) Ki-67-positive nuclei detected in poorly differentiated carcinoma, including atypical mitosis. (C, D) Poorly differentiated carcinoma exhibiting CD68-positive cells within the tumor stroma. Moderately differentiated carcinoma exhibiting strong (E), moderate (F), weak (G) granular cytoplasmic staining for COX-2.

positive nuclei than well-differentiated tumors ($P = 0.01$).

The expression of macrophages (CD68-positive cells)
CD68-positive cells were constitutively observed in all tumors (Fig. 1C,D). Mean macrophage density was 8.25 in *in situ* tumors, 8.21 in well-differentiated OSCCs, 8.82 in moderately differentiated carcinomas, and 10.66 in poorly differentiated carcinomas. With respect to the pattern of distribution, macrophages were clustered or diffusely distributed along the inflammatory infiltrate, with no significant difference in mean macrophage density ($P = 0.51$).

The expression of COX-2
The immunohistochemical expression of COX-2 was investigated in the cytoplasm of epithelial tumor cells and the respective stroma. Expression of this protein

was detected in 82.76% of the tumors studied in both parenchyma and stroma, but predominated in the former. In the parenchyma, the mean proportion of COX-2 staining was 75.42%, being observed a granular staining pattern. COX-2 staining was mainly extensive and strong (score 3) in epithelial tumor cells of all OSCC cases, irrespective of the degree of differentiation (Fig. 1E–G). A COX-2 immunostaining score of 3 was observed in three (75.0%) of four *in situ* carcinomas, five (62.5%) of eight well-differentiated carcinomas, five (55.55%) of nine moderately differentiated carcinomas, and four (66.66%) of six poorly differentiated carcinomas. No significant difference in COX-2 expression was observed between tumors of different degrees of differentiation ($P = 0.89$). In the case of *in situ* carcinomas, marked staining was particularly observed in the intermediate layer. One *in situ* carcinoma, one well-differentiated carcinoma, and two moderately differentiated carcinomas were negative for COX-2. Analysis of the tumor stroma was only descriptive and COX-2 staining was mainly detected in inflammatory interstitial cells and, occasionally, in endothelial cells.

Comparison between the expression of Ki-67, CD68 and COX-2

Analysis of the immunohistochemical expression of Ki-67, CD68 and COX-2 revealed no significant correlation between these biological markers (Ki-67xCD68, $P = 0.75$; COX-2xCD68, $P = 0.67$; Ki-67xCOX, $P = 0.76$). The histological classification of the tumors and mean score of each marker are shown in Table 1.

Table 1 Histological characteristics of the cases studied and results of the immunohistochemical analysis of the biological markers

Case	*Histological classification*	*Ki-67 index (%)*	*CD68 + density*[a]	*COX-2 (score)*
1	IS	14.14	96.50	3
2	IS	47.52	ND	3
3	IS	34.25	55.82	3
4	IS	18.89	95.29	0
5	WD	56.01	ND	3
6	WD	48.01	71.44	3
7	WD	26.12	40.23	1
8	WD	23.50	125.65	3
9	WD	31.89	73.55	3
10	WD	27.12	61.87	3
11	WD	17.35	68.08	0
12	WD	45.17	133.87	ND
13	MD	12.40	10.50	3
14	MD	36.47	70.35	2
15	MD	27.25	44.47	3
16	MD	43.95	130.34	3
17	MD	37.24	83.73	3
18	MD	30.53	95.87	3
19	MD	50.37	77.22	3
20	MD	52.43	65.31	0
21	MD	42.98	121.73	0
22	PD	18.83	153.96	2
23	PD	53.85	145.70	3
24	PD	23.15	93.19	3
25	PD	20.86	71.81	3
26	PD	38.68	91.44	ND
27	PD	66.15	83.76	3

IS, *in situ* carcinoma; WD, well-differentiated carcinoma; MD, moderately differentiated carcinoma; PD, poorly differentiated carcinoma; 0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining; ND, not determined.
[b]Mean number of positive cells per tissue area.

Discussion

According to the histological grading performed by the WHO, most OSCC cases of this study presented a moderate degree of differentiation, followed by well-differentiated tumors. These results agree with those reported by Huang et al. (26), who used the WHO classification to study 172 OSCCs of the buccal mucosa.

Ki-67 has been an excellent marker of cell proliferation (27–29). Its levels increase during the S phase, with maximum expression during the M phase. However, clone MIB-1 recognizes the antigen during all phases of the cell cycle (4, 5). The present results showed immunolocalization of Ki-67 mainly in cells of moderately and poorly differentiated OSCCs, in agreement with other authors reporting high proliferative indices for oral neoplasms with a poor prognosis (30). In contrast, our results disagree with those reported by Kuratomi et al. (31), who observed no correlation between the proliferative index measured by Ki-67 immunostaining and the degree of differentiation of OSCCs of the gingiva. The observation of a larger number of Ki-67-labeled cells in moderately differentiated OSCCs compared with poorly differentiated tumors might be explained by the larger number of moderately differentiated cases in the present sample.

Macrophages are the main inflammatory cell component of tumors and can exert contradictory functions (32, 33). When adequately activated, macrophages exert an important action against tumor cells as along as their precursors bind to endothelial cells, an event mediated by the expression of chemokines (34) and adhesion molecules (35, 36). However, TAMs may favor the growth and dissemination of tumors by mediating the expression of growth factors and MMPs, promoting angiogenesis and suppressing the adaptive immune response (1, 9, 37). In the present study, a high density of TAMs was observed in all tumors, irrespective of the histological grade of malignancy. Our findings agree with those reported by Hasselblom et al. (38) for lymphomas, who observed no association between the presence of CD68-positive cells and prognostic factors. In contrast, Valković et al. (39) and Kawai et al. (40) also observed high levels of TAMs in breast and lung cancer, respectively, and associated this finding with tumor prognosis. As OSCCs are poorly immunogenic and highly immunosuppressive (41), TAMs probably play an important role in these tumors.

The present results revealed high expression of COX-2 in all tumors, irrespective of the histological grade of malignancy. However, the question whether COX-2 is associated with tumor prognosis remains controversial. Some authors mainly associate COX-2 expression with the initiation of tumorigenesis (42, 43), whereas others preferentially relate its expression to distant dissemination (26). In the case of oral cancer, the tumor prognosis

does not depend on COX-2 levels as the latter are not associated with the degree of differentiation or tumor stage (16).

In the present study, COX-2 was also detected in the interstitial cells of tumors of different degrees of malignancy, as well as in the wall of blood vessels located close to the OSCCs. Although macrophages are the main secretory cells of the tumor interstitial component (12), other inflammatory cells, fibroblasts, and endothelial cells also produce COX-2 (42, 44). Macrophages secrete COX-2 and represent functional cells that initiate mitogenesis after being stimulated by prostaglandins (45, 46). Prostaglandin E_2 is the main subproduct of COX-2 that has been associated with malignant transformation. We believe that the expression of this protein by macrophages strengthens the anti-apoptotic and proliferative properties of the tumor (14). Furthermore, it is possible that the strong immunoexpression of COX-2 observed in *in situ* carcinomas of the present sample might be related to both the cancer-initiating and repair function of this protein, as Sheehan et al. (15) suggested that the expression of COX-2 by macrophages might be associated with tumor repair.

In the present study, no correlation was observed between the immunoexpression of COX-2 and Ki-67. Similar results have been reported by Yoshida et al. (29) for ovarian tumors. The suggestion of these authors that, in this case, COX-2 is involved in pathogenic mechanisms other than the promotion of cell proliferation might explain our results. As the interference of COX-2 with tumor growth and dissemination is not limited to the stimulation of mitogenesis (17), other mechanisms such as the induction of MMP expression seem to be involved in the process of oral carcinogenesis mediated by this oncoprotein. Mao et al. (47), studying lung tumors in active smokers, observed a decrease in the Ki-67 index after the administration of a COX-2 inhibitor but no significant reduction in its immunohistochemical expression. In the present study, TAM density was also not correlated with Ki-67 immunoexpression. Similar results have been reported by Hasselblom et al. (38) for B cell lymphomas.

There was also no correlation between the expression of COX-2 and the presence of TAMs. We believe that in OSCCs the expression of COX-2 is more related to epithelial tumor cells rather than to macrophages. In contrast, Bianchini et al. (32), investigating melanomas, considered the expression of COX-2 by macrophages to be more important in these tumors. Thus, we suggest that in OSCCs, in addition to COX-2, TAMs induce the expression of other molecules such as proangiogenic factors or those related to extracellular matrix degradation.

In conclusion, imunolabeling for Ki-67 was directly correlated with less-differentiated tumors, suggesting that this marker may contribute to understand the biological behavior of OSCC, and help to distinguish risk groups of OSCC. Furthermore, the lack of correlation between Ki-67, COX-2 and CD68 indicates that the latter two markers may play a pivotal role in oral carcinogenesis. However, further studies are needed to clarify their contribution for cell proliferation and tumor differentiation.

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