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# Morphometric analysis of the tumor associated tissue eosinophilia in the oral squamous cell carcinoma using different staining techniques

**S.C.M. Lorena[1], R.G. Dorta[1], G. Landman[2], S. Nonogaki[3] and D.T. Oliveira[1]**

[1]Department of Stomatology, Area of Pathology, Bauru School of Dentistry – University of São Paulo - Brazil and

[2]Department of Pathology, Cancer Hospital, São Paulo, Brazil and [3]Adolfo Lutz Institute, Pathology Division, São Paulo, Brazil

**Summary.** In a previous study, we found tumor-associated tissue eosinophilia (TATE) to be a favourable prognostic indicator for oral squamous cell carcinomas. Special techniques such as autofluorescence or immunohistochemistry are reported to be sometimes necessary to detect the presence of intact and degranulating eosinophils within the tumors. The aim of this study was to compare the number of eosinophils identified routinely with hematoxylin and eosin stain and by immunohistochemistry in oral squamous cell carcinomas with TATE. Thirty specimens of oral squamous cell carcinoma of the tongue, floor of the mouth, retromolar area and inferior gingiva with TNM stages II and III were used for histopathological analysis. Three-micrometer sections were stained with hematoxylin and eosin and immunohistochemically with monoclonal anti-human granulocyte-associated antigen using a standard streptavidin-biotin-peroxidase complex technique. The number of eosinophils/mm$^2$ in the invasive front of the tumors was automatically quantified in a x400 field using an image computer analyser. Univariate statistical analysis was carried out using Student's t test. The computer-assisted morphometric results showed that there was no statistically significant difference ($p>0.05$) in the number of eosinophils/mm$^2$ identified by hematoxylin and eosin or immunostaining technique in oral squamous cell carcinomas with TATE. This result suggests that hematoxilyn and eosin routine stain is a useful technique for measuring eosinophils in squamous cell carcinoma with eosinophilic tumor infiltration.

**Key words:** Eosinophils, Squamous Cell carcinoma, Eosinophilia, Oral cancer

*Offprint requests to:* Dra. Denise Tostes Oliveira, Faculdade de Odontologia de Bauru, Área de Patologia, Alameda Octávio Pinheiro Brisolla, 9-75 CEP 17012-901, Bauru, São Paulo, Brazil. Fax: 0055 21 14 2234679. e-mail: d.tostes@fob.usp.br

## Introduction

Eosinophils are a sub-population of granulocytes involved in the pathogenesis of important diseases, including some types of human cancer (Samoszuk, 1997). Tumor-associated tissue eosinophilia (TATE) in malignancies has been recognised for almost 100 years and has been described in many carcinomas located in different sites as nasopharynx, larynx/pharynx, gastrointestinal tract, lung, external genitalia, esophagus and oral cavity (Kodama et al., 1984; Lowe and Fletcher, 1984; Goldsmith et al., 1987, 1992; Looi, 1987; Samoszuk, 1997; Blumenthal et al., 2000; Ohashi et al., 2000). The role of eosinophils in oral and other cancers remains uncertain.

Numerous studies have associated TATE with favourable prognosis (Goldsmith et al., 1987, 1992; Thompson et al., 1994; Nielsen et al., 1999; Fernandez-Acenero et al., 2000; Dorta et al., 2002) as well as unfavourable prognosis (Horiuchi et al., 1993; Van Driel et al., 1999; Wong et al., 1999), or even with no influence on patients' outcome (Looi, 1987; Sassler et al., 1995). However, the exact role of tumor-associated tissue eosinophilia (TATE) as a prognostic factor in epithelial tumors is inconclusive.

Although intact eosinophils can usually be easily recognised in tissue sections of tumors that are stained with hematoxylin and eosin, sometimes these granulocytes assume an uncommon morphology specially in fibrous tissue or inflammatory infiltrate making their recognition in routinely stained sections very difficult (Samoszuk, 1997). In these situations, special techniques such as autofluorescence or immunohistochemistry are needed to detect the presence of intact and degranulating eosinophils within the tumors (Samoszuk, 1997; Blumenthal et al., 2000).

In a previous study (Dorta et al., 2002) we found tumor-associated tissue eosinophilia (TATE) to be an independent favourable prognostic indicator for oral squamous cell carcinomas with TNM stages II and III, located on the tongue, oral floor, retromolar area and

inferior gingiva. Then, we proposed using part of the same sample to compare the presence of the eosinophils stained routinely with hematoxylin and eosin and those immunohistochemically identified in oral squamous cell carcinomas with tumor-associated tissue eosinophilia.

## Material and methods

Thirty surgically specimens of primary oral squamous cell carcinoma of the tongue, floor of the mouth, retromolar area and inferior gingiva, with TNM stages II and III, presenting TATE were used for histopathological analysis. All the patients were submitted to surgical treatment from March 1970 to December 1992 at the Cancer Hospital A.C. Camargo, Fundação Antonio Prudente, in São Paulo, Brazil. None of these patients received radio, chemotherapy or other previous treatment prior to surgery. Moreover, tumors with extensive ulceration or necrosis and patients presenting other simultaneous primary tumors were excluded from the sample. Clinical data, therapy and recurrence were obtained from the medical records. Tumor-associated tissue eosinophilia was previously determined by Dorta et al. (2002) and ranged from 84 to 392 per square milimeter in oral squamous cell carcinomas with intense eosinophilic infiltration.

Histological and immunohistochemical studies were performed on paraffin sections of representative portions of the tumors in order to identify eosinophil distribution in the tissue. Formalin-fixed-3 $\mu$m sections were used for hematoxylin and eosin staining or immunohistochemistry analysis with monoclonal anti-human granulocyte-associated antigen (CD15 clone C3D-1, DAKO ref M0733, Denmark) using a standard streptavidin-biotin-peroxidase technique. The sections were deparaffinized and rehydrated in decreasing ethanol concentrations. After antigen retrieval using 10 mM citrate buffer, pH 6.0, in pressure cooker during five minutes (Norton et al., 1994), endogenous peroxidase activity was blocked by incubation in 3% $H_2O_2$ for 20 minutes. The sections were incubated overnight at 4 °C, with primary antibody 1:400 in bovine serum albumin (BSA) solution to block nonspecific reaction. The antigen-antibody reaction was detected using streptavidin-biotin-based detection kit (StreptABComplex HRP, Duet, Mouse/Rabbit, Dako, ref K0492, Denmark) and visualised using 3,3-diaminobenzidine tetrahydrochloride (DAB/SIGMA, ref D-5637,USA). Sections were counterstained with Harris hematoxylin before being dehydrated and coverslipped. Appropriate positive and negative controls were included in each run.

Quantitative, computer-assisted morphometric analysis of tumor sections was performed by two observers (SCML and DTO). The eosinophils identified by hematoxylin and eosin or immunostaining with CD15 were automatically counted in a x400 field, using a computer image (Image Pro Plus 4.0). A total of twenty-five images of the invasive front tumor in each slide was captured digitally using a CCD Camera that was attached to a light microscope. The slide image captured by the computer had a real field on the TV screen of 0.015875mm$^2$. The average number of eosinophils/mm$^2$ obtained in squamous cell carcinomas stained by hematoxylin and eosin or immunohistochemically detected was then statistically compared by univariate analysis carried out using Student's t test.

## Results

The main clinical features of our series of patients are summarized in Table 1. Ages ranged from 30 to 81 years old (mean, 55.5 years). Based on the UICC (International Union Against Cancer) classification of oral cavity carcinomas, tumors were classified as T2 (70%), T3 (30%), N0 (60%) and N1 (40%). Blood eosinophilia (values above 5%) was detected in 10 patients (33.33%).

An extensive eosinophilic inflammatory infiltrate was observed in the invasive front of the squamous cell carcinomas stained with hematoxylin and eosin as well as positively stained with CD15 (Fig. 1). The intense brown staining of CD15 was localized within apparently intact eosinophils. Interestingly, the eosinophils were found intimately associated with tumor cells or with a strong lymphoplasmatic cell infiltration. In all cases,

**Table 1.** Clinical findings, treatment and recurrence of the patients with primary squamous cell carcinoma.

| CLINICAL FEATURES | | NUMBE OF CASES | PERCENTAGE (%) |
|---|---|---|---|
| Age | ≤ 65 years | 25 | 83.33 |
| | >65 years | 5 | 16.66 |
| Gender | Male | 25 | 83.33 |
| | Female | 5 | 16.66 |
| Ethnic group | Caucasian | 26 | 86.66 |
| | Non-Caucasian | 4 | 13.33 |
| Location | Oral tongue | 22 | 73.33 |
| | Floor of the mouth | 5 | 16.66 |
| | Retromolar area | 2 | 6.66 |
| | Inferior gingiva | 1 | 3.33 |
| Tobacco | Yes | 25 | 83.33 |
| | No | 4 | 13.33 |
| | Unknown | 1 | 3.33 |
| Alcohol | Yes | 22 | 73.33 |
| | No | 7 | 23.33 |
| | Unknown | 1 | 3.33 |
| Tumor stage | II | 13 | 43.33 |
| | III | 17 | 56.66 |
| Neck dissection | Ipsilateral | 25 | 83.33 |
| | Bilateral | 5 | 16.66 |
| Postoperative RXT | Yes | 12 | 40 |
| | No | 18 | 60 |
| Recurrence | Yes | 8 | 26.66 |
| | No | 22 | 73.33 |

RXT: radiotherapy

tumor cells and lymphocytes were negative for CD15.

Table 2 shows the data concerning eosinophil count in our oral squamous cell carcinoma sample. These morphometric results showed that there was no statistically significant difference ($p>0.05$) in the average number of eosinophils/mm$^2$ identified by

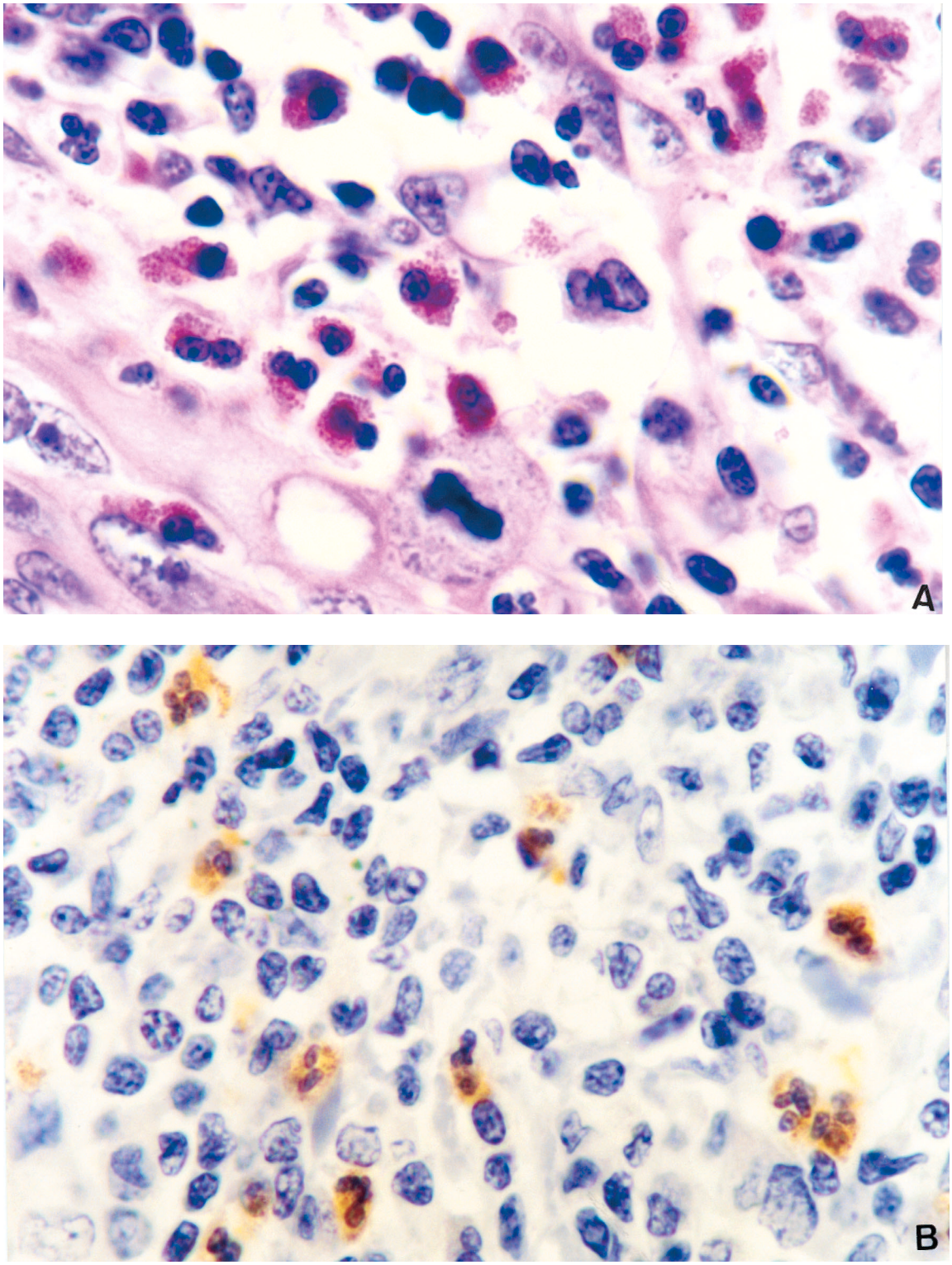


**Fig. 1.** Intense eosinophilic inflammatory infiltrate in invasive front of the oral squamous cell carcinomas identified with hematoxylin and eosin **(A)** or immunostaining with CD15 **(B)**. A, x 330; B, x 312

hematoxylin and eosin or by immunohistochemistry in oral squamous cell carcinomas with TATE.

## Discussion

The functional role of eosinophils in human cancer as well as the eosinophilotactic compounds remains uncertain (Samozusk, 1997). It has been suggested that the eosinophils play a role in the host immune response, specifically directed against the tumor cells. On the other hand, there are reports implying the participation of these cells in a more general type of inflammatory response related to wound healing and tissue remodelling prompted by the growing tumor (Samozusk, 1997; Nielsen et al., 1999; Blumenthal et al., 2000).

Recently, Dorta et al. (2002) demonstrated that the intense tumor-associated tissue eosinophilia is an independent favourable prognostic factor for oral squamous cell carcinomas.

In order to find out if the CD15 antibody provides relevant visualization of the eosinophils in oral squamous cell carcinomas with TATE, we decided, using part of the sample studied by Dorta et al. (2002), to quantify by morphometric analysis the positive eosinophilic granulocytes in invasive front tumor.

The CD15 antigen is recognized by a family of monoclonal antibodies and is raised against human granulocytes (Dorfman et al., 1986). It has been shown to recognize the glycolipid and glycoprotein components of the granulocyte cell surface (McCarthy et al., 1985). The CD15 antibody is also positive for another subpopulation of granulocyte, the neutrophils. The presence of the neutrophilic inflammatory infiltrate in malignant tumors is mainly associated with extensive ulceration and/or necrosis but, in our tumor sample, it was one of the criteria of exclusion.

Degranulating eosinophils have been identified by immmunohistochemistry with anti-eosinophil peroxidase antibody in many diseases, including cancers (Blumenthal et al., 2000). Eosinophil peroxidase is an intracellular enzyme that is released from eosinophils when they degranulate. This antibody can recognize intact eosinophils as well as the "footprints" of eosinophils that have previously undergone degranulation in blood vessels adjacent to the tumor (Samoszuk, 1997). Since the objective of the our current study did not consist in determining of the biological role of eosinophil, but just in their detection in oral squamous cell carcinomas, we decided to use an anti-granulocyte monoclonal antibody that provided an excellent immunostaining for intact eosinophils (Fig. 1), including those within the vessels.

In our sample, the number of eosinophils/mm$^2$ identified was not statistically different between the tumors stained by hematoxylin and eosin or immunostaining with CD15. However, the detection of the eosinophils, from some tumors, was lower by immunohistochemistry than by routinely hematoxylin and eosin stain (Table 2). It probably occurred because when a monoclonal antibody as CD15 is used, it reacts with only one epitope on the molecule, resulting in fewer antibody molecules being bound to the antigen and subsequently detected by the labeling method (Polak and Noorden, 1997). Moreover, our tumor samples were fixed in formalin and paraffin embedded from 1970 to 1992 and the alteration of the epitope of our interest on the antigen molecule may have occurred by fixation or processing, resulting in no staining (Leong and Gilham, 1989).

Confronting the results found by Dorta et al. (2002) and the current results related to number of eosinophils/mm$^2$ in squamous cell carcinomas with intense eosinophilic inflammatory infiltrate, we verified that the average number of eosinophils was higher in our tumor sample. This disagreement could be explained by comparing the total tissue tumor area used for morphometric analysis. For each specimen Dorta et al. (2002) evaluated 75 random microscopic tumor fields (covering a total area corresponding to 1,3246875mm$^2$ per tumor) and eosinophils were assessed through the entire depth, including malignant cells and tumor stroma. In our current study the eosinophils were quantitatively assessed just in the invasive front tumor covering a total area of 0,396875mm$^2$ per oral squamous cell carcinoma.

These results show the difficulty of comparing TATE in squamous cell carcinomas, even when part of the same sample is used. They also reinforce the observation that measurement of eosinophils in areas previously determined such as the invasive front tumor, where important carcinoma-stromal interactions occur, could not reflect precisely the occurrence of TATE in malignant tumors (Dorta et al., 2002).

Most pathologists do not routinely count eosinophils in malignant tumors although this determination in hematoxilyn and eosin sections can be easily performed. Our results support the evidence that no special staining techniques are necessary for the measurement or identification of TATE in oral squamous cell carcinomas. Eosinophilic tumor infiltration seems to define a better prognosis in oral cancer (Goldsmith et al., 1987, 1992; Dorta et al., 2002) and eosinophil counts could become, in the future, useful for therapeutic approaches in this subset of patients.

**Table 2.** Number of eosinophils/mm$^2$ in oral squamous cell carcinomas with TATE.

| GROUP | MEAN | STANDARD DEVIATION |
|---|---|---|
| Hematoxylin & Eosin | 442.56* | 302.54 |
| Immunohistochemistry with CD15 | 307.75* | 233.17 |

*:no statistically significant difference for p>0.05

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