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jbpm@sbpc.org.br, adagmar.andriolo@gmail.com

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Immunohistochemical study of macrophages subpopulations associated with squamous cell carcinoma of the tongue, with and without metastasis

Estudo imuno-histoquímico das subpopulações de macrófagos associados a tumores em carcinoma epidermoide de língua, com e sem metástase

Natália G. Barbosa; Melka C. Sá; Leão P. Pinto; Roseana A. Freitas

Universidade Federal do Rio Grande do Norte (UFRN).

ABSTRACT

Introduction: Stromal cells interact with tumor cells and play an important role in cancer invasion and metastasis. Tumor-associated macrophages (TAMs) can exhibit M1 phenotype, important immune effector cells, or M2 phenotype, able to suppress the function of M1 macrophages and influence angiogenesis and tissue repair. The CD68 antibody recognizes M1 and M2 macrophages, whereas CD163 antibody is specific only to identify M2 macrophages. **Objective:** To investigate the presence of TAMs in a number of cases squamous cell carcinoma (SCC) of the tongue by associating it with the occurrence of metastasis. **Material and methods:** Immunohistochemistry was used to evaluate the immunopositivity for CD68 and CD163 in 27 cases of SCC of the tongue. **Results:** The percentage of CD68 positive macrophages was higher than CD163 positive macrophages in all specimens studied. Comparing CD163 and CD68 immunostaining in the studied groups, it was observed that cases without metastasis had a higher percentage of CD68 positive cells in relation to CD163 positive cells, which was statistically significant. **Conclusion:** Considering these results, there was a predominance of M1 macrophages in SCC of the tongue cases without metastasis, suggesting the influence of these cells in clinical behavior of the lesion.

Key words: squamous cell carcinoma; macrophages; cancer of the tongue; immunochemistry.

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the eighth most common cancer worldwide⁽¹⁾ and leads to a high degree of morbidity with functional and aesthetic impairment⁽²⁾. O OSCC represents more than 90% of malignant tumors in this region⁽³⁾, and for Brazil was estimated in 2014, 11,280 new cases of oral cavity cancer in men and 4,010 in women⁽⁴⁾. Despite advances in surgical treatment, the prognosis of this type of cancer has not improved in the last 30 years⁽⁵⁾.

Stromal cells interact with the neoplastic cells and play an important role in tumor progression⁽⁶⁾. Macrophages are widely distributed throughout the body and participate in the inflammatory reactions against foreign bodies and microbial

invasions. In neoplastic growth, the macrophages are often found in the tumor stroma and are called tumor-associated macrophages (TAMs)^(7,8).

According to tumor microenvironment stimulation, the macrophages may be polarized in two different phenotypes: M1 or M2. Interferon- γ (IFN- γ) and lipopolysaccharide mediate the M1 macrophages activation, while the interleukins (IL)-4, IL-10 and IL-13 mediate the M2 activation⁽⁹⁾. Classical M1 macrophages are considered potent effector cells that may destroy tumor cells by producing nitric oxide and reactive oxygen; M2 macrophages are characterized by their ability to inhibit the cytotoxic and inflammatory functions of M1 macrophages, and also is involved in angiogenesis and tissue repair and remodeling⁽¹⁰⁾. The CD68 antibody, which recognizes both M1 and M2 macrophages, has been used to identify them⁽¹¹⁾. On the

other hand, CD163, a glycoprotein belonging to the hemoglobin scavenger receptor group, is a specific antigen for identification of M2 macrophages⁽¹²⁾.

TAMs have been studied for years as a major cellular component in human cancers, but there is no consensus about its role in prognosis, tumor growth and in metastasis. The increase in CD163 expression was significantly associated with the low overall survival rate of several cancers^(13, 14). In OSCC, positive correlation of macrophage infiltration into neoplastic stroma and invasion front in a higher histological grade were observed⁽³⁾, and when T3 and T4 tumors were investigated, the amount of CD68 positive macrophages in OSCC specimens with metastasis was significantly higher than in those without metastasis and in the control group⁽¹⁵⁾.

Based on this, our study aimed to investigate the presence of M1 and M2 macrophages in squamous cell carcinoma (SCC) of the tongue samples through the immunohistochemical expression of CD68 and CD163, respectively. In addition, the presence of these populations of macrophages was associated with the presence/absence of metastasis, in order to clarify the role of these inflammatory cells in the biological behavior of SCC of the tongue.

MATERIAL AND METHODS

Sample

The sample selected for this study comprised 27 cases of SCC of the tongue, registered and diagnosed at the Laboratory of Anatomic Pathology in the Discipline of Oral Pathology, Department of Dentistry in the Universidade Federal do Rio Grande do Norte (UFRN), Natal (RN). We collected clinical data regarding the presence or absence of metastasis, from the medical records of the selected cases. The study was approved by the Research Ethics Committee of UFRN under opinion N° 910.190.

Morphological study

For morphological analysis, the specimens were subjected to 5 mm thick sections and stained with hematoxylin and eosin (HE). Subsequently, the invasion front in each case was analyzed and

classified as to the invasion pattern: a) compressive, infiltrating and well-defined margins; b) infiltrating, solid cords, bands or strands; c) small groups or cord of more than 15 cells; d) marked and widespread cell dissociation in small groups ($n < 15$) and/or individual cells; and e) intensity of lymphoplasmocytic infiltration (marked, moderate, mild or absent). The analysis of these parameters was analyzed according to the Deep Invasive Margins Grading System, developed by Bryne⁽¹⁶⁾, which indicates the clinical behavior of the lesion by evaluating four histological parameters (degree of keratinization, nuclear pleomorphism, invasion pattern and lymphoplasmocytic infiltration) in tumor invasion front.

Immunohistochemical method

All SCC of the tongue specimens selected underwent 3 mm thickness histological sections, which were mounted on glass slides previously cleaned and degreased coated with 3-aminopropyltriethoxysilane (Sigma Chemical CO, St Louis, MO, USA)- adhesion. Then, the sections were dewaxed with xylene and hydrated into ethanol, followed by washing in distilled water and antigen retrieval, then were incubated with anti-CD68 and anti-CD163 primary antibody (**Table 1**). The sections were then washed twice in Tween 20 solution at 1% in Tris (hydroxymethyl) aminomethane hydrochloride (TRIS-HCL) (pH 7.4) and incubated for 30 minutes in streptavidin-horseradish peroxidase (HRP) complex (Streptavidin-HRP, Dako North America Inc., Carpinteria, CA, USA), at room temperature. The peroxidase activity was visualized by immersion of tissue sections in chromogen solution of 3,3-diaminobenzidine (Liquid DAB + Substrate, Dako North America Inc., Carpinteria, CA, USA), which resulted in a brownish color reaction product. Subsequently, the sections were counterstained with Mayer's hematoxylin. The negative control consisted of the replacement of the primary antibody to bovine serum albumin (BSA) at 1% in buffer solution.

Immunohistochemical study and statistical analysis

To evaluate the CD68 and CD163 antibodies expression we considered positive all cells that exhibited brownish color,

TABLE 1 – Specificity, catalog number, manufacturer, dilution, antigen retrieval, and incubation time of primary antibodies to be used in the study

Specificity	Catalogue number	Manufacturer	Dilution	Antigen retrieval	Incubation time
CD68 (KP1)	sc-20060	Dako	1:50	Citrate pH 6.0, Pascal 121°C, 3 minutes	60'
CD163 (10D6)	ab74604	Abcam	Prediluted	Citrate pH 6.0, Pascal 121°C, 3 minutes	60'

regardless of the intensity in the cytoplasmic or nuclear region. Therefore, the CD68 and CD163 expression was examined by two independent researchers using light microscope Olympus CH30 (Olympus Japan Co, Tokyo, Japan), 200× increased. The percentage of immunopositive cells for both markers was recorded semi-quantitatively, in the inflammatory infiltrate of the invasion front, in consecutive fields to the full extent. Thus, analysis of the percentage of macrophages in relation to other cells of the inflammatory infiltrate was classified as follows: 0 when less than 10%; 1 between 11%-50%; and 2 and when greater than 50%.

Once the collection of clinical data, histopathological and immunohistochemical, were performed, the results were then subjected to Pearson's chi-square statistical parametric test and Mann-Whitney nonparametric test, to verify associations between variables, according to their nature. To check which immunomarker is more expressed in each group (with and without metastasis), the Wilcoxon test was used. For all analyzes, we considered the significance level of $p < 0.05$.

RESULTS

Morphological features

From the 27 selected patients, 20 (74%) showed no metastasis, and seven (26%) showed metastasis. Eleven cases (40.7%) had an infiltrative invasion pattern, through solid cords, bands or strands. The second more frequent invasion pattern was through small groups of cells (25.9%), followed by dissemination and decoupling in small groups and/or individual cells (18.5%) and the compression pattern with well-defined infiltrating margins (14.8%). The inflammatory infiltrate was present in all cases, in

different intensities. Moderate infiltration was the most common (48.1%), followed by the striking number of inflammatory cells (37%). The minority of patients had mild inflammatory infiltrate (14.8%). In analyzing for the presence or absence of metastasis, most cases of metastasis presented an invasion pattern with groups and cords with fewer cells (57.1%); while for those without metastasis, the predominant invasion pattern was through solid cords a large number of cells (50%). Regarding the inflammatory component, the metastatic cases showed more marked infiltrate (57.1%) than those without metastasis, in which the moderate infiltrate was most prevalent (50%) (**Table 2**).

Immunoexpression for CD68 and CD163

The immunopositive cells for CD68 and CD163 distributed predominantly in the stroma, surrounding or within the neoplastic cell nests (**Figures 1 and 2**). All cases showed immunoexpression for CD68. Most cases (59.3%) exhibited score 2, that is, more than 50% of the immunostained cells, while 40.7% were ranked with a score (11% to 50% of immunostained cells). Regarding the CD163, five cases (18.5%) scored 0 (0% to 10% of immunostained cells), and the majority of cases (59.3%) classified as score 1, and 14.8%, scored 2 (**Table 3**). We did not observed statistically significant differences in immunohistochemical marking of CD68 and CD163 between without and with metastasis ($p = 0.45$ and $p = 0.09$, respectively) (**Table 4**). By comparing the immunostaining of CD68 and CD163 for each studied group (**Table 5**), it was observed that the group without metastasis showed higher amounts of CD68 positive cells in relation to CD163 positive, this result is statistically significant ($p < 0.001$). This finding was not observed in the group with metastasis, which showed equivalent proportion of CD68 and CD163 positive cells.

TABLE 2 – Absolute and relative distribution and statistical significance of the variables analyzed for the presence of metastasis

	Without metastasis <i>n</i> (%)	With metastasis <i>n</i> (%)	<i>p</i> *	Total <i>n</i> (%)
Type of invasion				
Compressive, well-defined infiltrating borders	3 (15)	1 (14.3)	0.15	4 (14.8)
Infiltrative, solid cords, bands or strands	10 (50)	1 (14.3)		11 (40.7)
Small groups or solid cords, bands or strands	3 (15)	4 (57.1)		7 (25.9)
Marked and widespread dissociation in small groups and/or in singles cells	4 (20)	1 (14.3)		5 (18.5)
Inflammation				
Absent	0 (0)	0 (0)	0.29	0 (0)
Mild	4 (20)	0 (0)		4 (14.8)
Moderate	10 (50)	3 (42.9)		13 (48.1)
Marked	6 (30)	4 (57.1)		10 (37)
Total	20 (100)	7 (100)		27 (100)

*: Pearson's Chi-square test; $p \leq 0.05$.

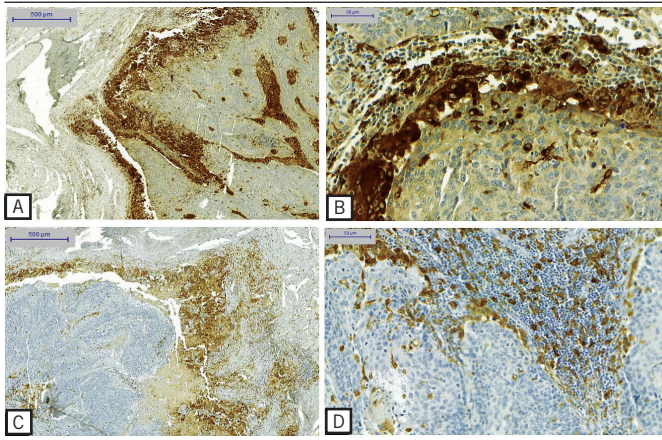


FIGURE 1 – SCC of the tongue

Intense immunohistochemical expression of CD68 (A and B) and CD163 (C and D), showing compressive invasion pattern, with outlined infiltrating border adjacent to abundant inflammatory infiltrate (Panoramic Viewer, Advance).

SCC: squamous cell carcinoma.

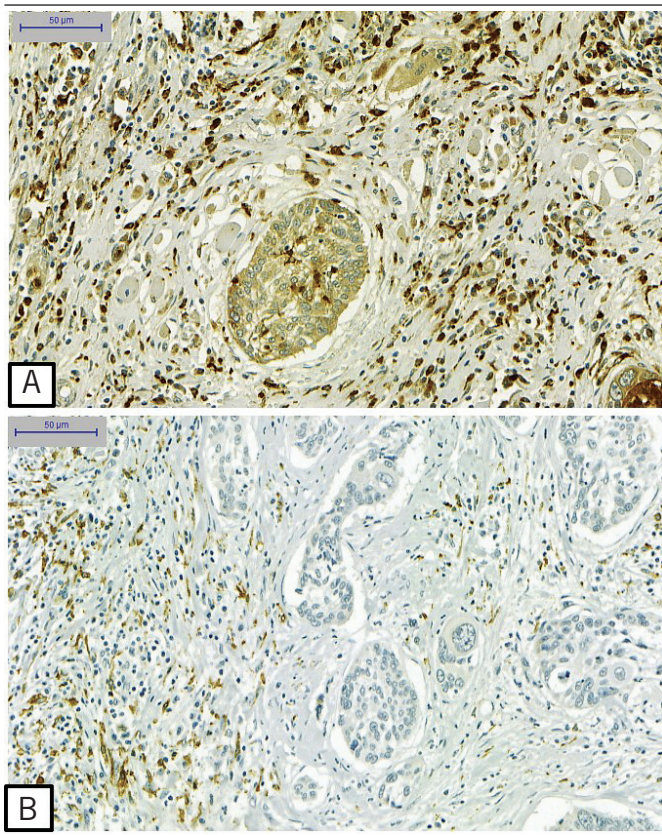


FIGURE 2 – SCC of the tongue

Mild immunohistochemical expression of CD68 (A) and CD163 (B), showing invasion pattern with dissociation in small groups and/or single cells adjacent to mild inflammatory infiltrate (Panoramic Viewer, Advance).

SCC: squamous cell carcinoma.

TABLE 3 – Absolute and relative distribution of immunohistochemical expression of CD68 and CD163 in relation to the presence of metastasis

	Without metastasis <i>n</i> (%)	With metastasis <i>n</i> (%)	Total <i>n</i> (%)
CD68			
Score 0 (0% to 10%)	0 (0.0)	0 (0.0)	0 (0.0)
Score 1 (11% to 50%)	9 (45.0)	2 (28.6)	11 (40.7)
Score 2 (> 50%)	11 (55.0)	5 (71.4)	16 (59.3)
CD163			
Score 0 (0% to 10%)	5 (25.0)	0 (0.0)	5 (18.5)
Score 1 (11% to 50%)	13 (65.0)	5 (71.4)	16 (59.3)
Score 2 (> 50%)	2 (10.0)	2 (28.6)	4 (14.8)
Total	20 (100.0)	7 (100.0)	27 (100.0)

TABLE 4 – Immunoreactivity difference for CD68 and CD163 in stromal cells between the groups with absent and present metastasis

Metastasis	<i>n</i>	CD68			CD163		
		Median	Q ₂₅ -Q ₇₅	<i>p</i> *	Median	Q ₂₅ -Q ₇₅	<i>p</i> *
Absent	20	2.00	1.00-2.00	0.45	1.00	1.00-1.00	0.09
Present	7	2.00	1.00-2.00		1.00	1.00-2.00	

*: teste de Mann-Whitney; $p \leq 0.05$.

TABLE 5 – Comparison of immunostaining for CD163 and CD68 in the groups with absent and present metastasis

Metastasis	<i>n</i>	CD68			CD163	
		Median	Q ₂₅ -Q ₇₅	Median	Q ₂₅ -Q ₇₅	<i>p</i> *
Absent	20	2.00	1.00-2.00	1.00	1.00-1.00	< 0.001
Present	7	2.00	1.00-2.00	1.00	1.00-2.00	0.08

*: Wilcoxon test; $p \leq 0.05$.

DISCUSSION

In recent decades, special attention has been given to the study of the molecular basis of oral carcinogenesis and to identification of molecular markers that may affect the patients prognosis⁽¹⁷⁾. The presence of TAMs is a striking feature of cancer-associated inflammation⁽¹⁸⁾. In this study, TAMs were observed in all cases analyzed, distributed in the tumor parenchyma, findings also observed by El-Rouby⁽³⁾ and Liu *et al.*⁽¹⁹⁾, suggesting the recruitment of these cells towards the tumor region and the consequent ability to modify the neoplastic process.

For morphological analysis of specimens of the study, we use an adaptation of the scoring system proposed by Bryne⁽¹⁶⁾.

The authors support the hypothesis that the molecular and morphological characteristics of the tumor-host interface (invasion front) better reflect tumor behavior than other tumor areas, since important molecular events in the spread of tumors, such as the gain and loss adhesion molecules, secretion of proteolytic enzymes, increased cell proliferation and early angiogenesis occur in the tumor-host interface (invasion front). We chose to evaluate the inflammatory infiltrate and the tumor invasion pattern, since we consider that there is an important interaction between type and intensity of the inflammatory infiltrate with the morphology, growth, invasion and spread of neoplastic epithelium, as suggested by Costa *et al.*⁽¹⁵⁾. We observed that metastatic cases have invasion pattern with groups and cords with fewer cells than in those without metastasis, which confirms Bryne findings⁽¹⁶⁾, that the invasion through small cell groups is associated with worse prognoses. Interestingly, when analyzing the intensity of the inflammatory infiltrate, we found that cases with metastasis showed marked infiltration, contrary to expectations as that cases with worse prognosis would present milder infiltrate than those with a better prognosis, reinforcing the fact that not only the quantity of inflammatory cells is important, but also the presence of cells with specific immunological profile is crucial for a good antitumor response, such as CD8, CD25 lymphocytes, natural killer cells and polarized macrophages, for example⁽¹⁵⁾.

In our study, numerous macrophages were observed along the invasion front, which indicates that such cells play an important role in the recognition and destruction of the tumor. The percentage of CD68 positive macrophages in SCC of the tongue specimens was slightly higher (most with score 2) than the percentage of CD163 macrophages (most with score 1). This result may reflect the fact that the CD68 antigen is expressed in both M1 and M2, and the CD163 macrophages is a specific marker for M2 phenotype^(15, 20).

In many immunohistochemical studies, it was observed that the number of TAMs CD68 and CD163 positive was associated with OSCC specimen prognosis. Lu *et al.*⁽²¹⁾ found that a significantly higher number of CD68 positive macrophages was observed in larger tumors, recurrent, with lymph node metastasis and advanced clinical stages. He *et al.*⁽²²⁾ found that CD68 and CD163 expression were significantly associated with the presence of lymph node metastasis, also

noting that patients with OSCC and CD163 overexpression had lower overall survival. The latter result was also observed by Fujii *et al.*⁽²⁰⁾. Based on these findings, we sought to associate the CD68 and CD163 immunoexpression to the presence of metastases in cases of SCC of the tongue of this study, but it was not detected statistically significant association.

Weber *et al.*⁽²³⁾ aimed to study the polarization of macrophages, i.e., which type of macrophage is predominant in OSCC, both in the parenchyma and in the tumor stroma, correlating it with the presence of metastasis. The authors observed greater immunostaining for CD163, in both the epithelium and tumor stroma in specimens with metastasis. They consider, then, a greater marking of CD163 in relation to CD68 in metastatic specimens would indicate a higher proportion of M2 macrophages in these cases. They also ratified that it is unclear if such event occurs by polarization in M2 phenotype within the tumor tissue, or if there is an increased infiltration of M2 macrophages already pre-polarized. In our study, we analyze the stroma in the invasion front of tumor and we observed an equivalent proportion of CD163 and CD68 positive macrophages in specimens with metastasis, however, when analyzing the cases without metastasis, we found a higher proportion of CD68 positive cells, and this result was statistically significant, corroborating the search above, in which M1 macrophages polarization was observed in cases without metastases, suggesting that these cells influence the clinical behavior of the lesion.

CONCLUSION

The results of this study indicate that TAMs are important cells in the development and progression of the SCC of the tongue, since they were present in all cases analyzed, in large quantities. In the specimens studied, there was a predominance of M1 macrophages in cases without metastasis, possibly indicating the influence of these cells in the clinical behavior of the lesion. It is suggested a thorough research on the immunological aspects involving the interaction of TAMs with the neoplastic epithelium, as it will provide a better understanding of the relationship between the inflammatory processes and the growth of malignant epithelial cells.

RESUMO

Introdução: As células do estroma interagem com as células neoplásicas e desempenham papel importante na invasão e na metástase do câncer. Os macrófagos associados ao tumor (TAMs) podem se apresentar com fenótipo M1, importantes células efetoras, ou fenótipo M2, capazes de suprimir a função dos macrófagos M1 e influenciar na angiogênese e no reparo tecidual. O anticorpo CD68 reconhece os macrófagos M1 e M2, enquanto o anticorpo CD163 é específico para a identificação apenas de macrófagos M2. **Objetivo:** Investigar a presença dos TAMs em uma série de casos de carcinoma epidermoide (CE) de língua, associando-a à ocorrência de metástase. **Material e métodos:** A técnica imuno-histoquímica foi utilizada para avaliar a imunopositividade ao CD68 e CD163 em 27 casos de CE de língua. **Resultados:** A porcentagem de macrófagos CD68 positivos foi maior do que a de macrófagos CD163 positivos em todos os espécimes estudados. Comparando a imunomarcagem de CD68 e CD163 nos grupos estudados, verificou-se que nos casos sem metástase havia maior proporção de células CD68 positivas em relação às CD163 positivas, o que foi estatisticamente significativo. **Conclusão:** Diante desses resultados, observou-se a predominância de macrófagos M1 em casos de CE de língua sem metástase, sugerindo a influência dessas células no comportamento clínico da lesão.

Unitermos: carcinoma de células escamosas; macrófagos; neoplasias da língua; imuno-histoquímica.

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MAILING ADDRESS

Roseana de Almeida Freitas

Universidade Federal do Rio Grande do Norte; Departamento de Odontologia; Avenida Senador Salgado Filho, 1.787; Lagoa Nova; CEP: 59056-000; Natal-RN, Brazil; Phone: +55 (84) 3215-4108; e-mail: roseanafreitas@hotmail.com.