

## MICROVESSEL AND MAST CELL DENSITIES IN MALIGNANT LARYNGEAL NEOPLASM

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**Abstract** – Laryngeal neoplasm contributes to 30-40% of carcinomas of the head and neck. Mast cells are normal connective tissue residents, well represented in the respiratory tract. Experimental evidence suggests that the growth of a tumor beyond a certain size requires angiogenesis, which may also permit metastasis. The aim of this study was to evaluate the correlation between mast cell density, microvascular density, histopathological type and histological grade. Our study included 38 laryngeal carcinomas as follows: adenoid cystic carcinoma (2 cases), malignant papilloma (2 cases) and squamous cell carcinoma (34 cases). The combined technique of CD 34-alcian blue safranin (ABS) was used to identify microvessel and mast cell density, which was quantified by the hot spot method. A significant correlation was found between both mast cell and microvascular density, and G1/G2 histological grade ( $p=0.002$  and  $p=0.004$ , respectively). Squamous cell carcinoma was significantly correlated with mast cell density ( $p=0.003$ ), but not with microvascular density ( $p=0.454$ ).

**Key words:** Larynx carcinoma; mast cells; microvessel density

### INTRODUCTION

Laryngeal cancer is the 14<sup>th</sup> most common cancer in the world (Kapil et al., 2005) and the 13<sup>th</sup> most common neoplasm in men in the European Union (Marioni, 2012). Despite improvements in the diagnosis and the treatment of this malignancy, the 5-year survival rate has not changed significantly between 1975 and 2013.

Several studies examined the involvement of angiogenic factors in the development of laryngeal tumors. Murray et al. (1997) found a strong correlation between angiogenesis and regional recurrence and

proposed blood vessel formation as an independent prognostic indicator. Beatrice et al. (1998) suggested that microvessel density is useful in the assessment of prognosis in the laryngeal squamous cell carcinoma. A direct correlation was found between increased tumor angiogenesis and T stage, histologic grade and a shorter survival rate in patients with laryngeal cancer by Kupisz et al. (1999).

Conflicting results concerning VEGF downregulation during head and neck tumorigenesis and no significant differences between microvessel density from adjacent normal epithelium premalignant lesions and tumor area were previously reported (Tae

et al., 2000). No difference in tumor angiogenesis in the head and neck area was found between metastatic and non-metastatic conditions. It was concluded from these studies that tumor angiogenesis is not a clinically useful marker in determining lymph node metastasis in these patients (Leedy et al., 2000).

There are two types of mast cells: connective tissue mast cells containing heparin and sulfated glycosaminoglycan, and mucosa-associated mast cells characterized by the absence of heparin and the presence of chondroitin sulfate. A significant correlation between microvessel density and mast cells was found in oral squamous cell carcinomas (Iamaroon et al., 1994; Mohtasham et al., 2010). These observations support the idea that mast cells promote tumor progression by angiogenesis upregulation

Mast cell role and angiogenesis in squamous cell carcinoma is still a debated topic. Mast cells induced neovascularization by producing angiogenic factors and substances with angiogenic properties: VEGF, tryptase, FGF, TNE, IL-8, heparin and histamine. In laryngeal carcinoma, the idea was put forward that laryngeal cancer cells and mast cells controlled angiogenic response by releasing vascular endothelial growth factor (VEGF) (Sawatsubashi et al., 2000). On the other hand, a decrease in mast cells in oral squamous cell carcinoma and no correlation between mast cell density and the clinical, microscopic characteristics of oropharyngeal squamous cell carcinomas (OSCC) were found (Oliviera-Neto et al., 2007).

In view of these controversial results, we undertook a study for the evaluation of the interrelation between mast cell and microvascular density, and the histopathological type and histological grade.

## MATERIALS AND METHODS

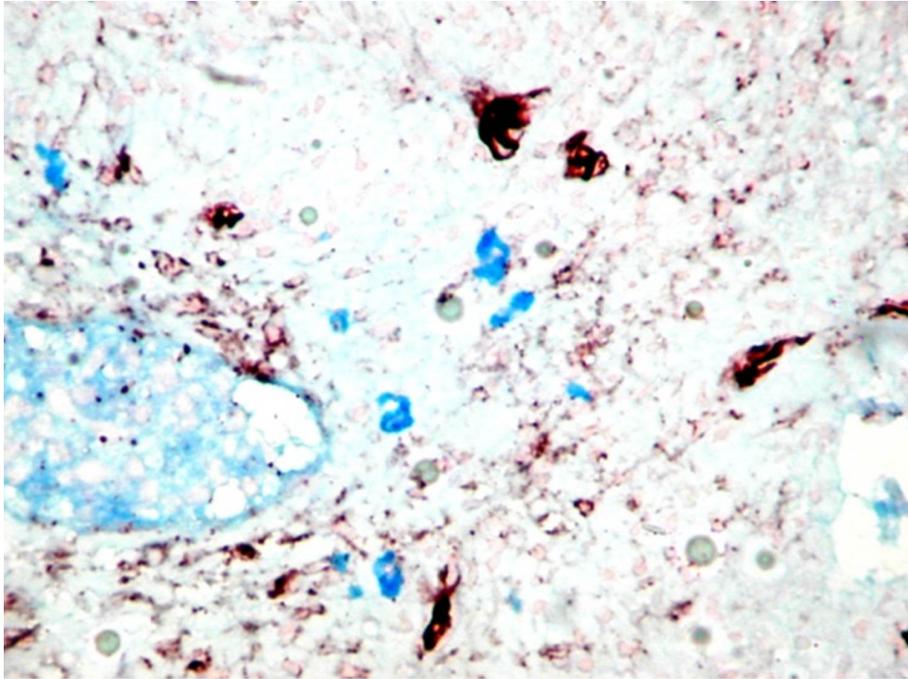
In this study we investigated 38 patients with bicocho-commissure laryngeal neoplasm. Biopsy samples were fixed in buffered formalin and paraffin embedded, according to the standard histological procedure. For each case, immunostaining with

CD34 antibody preceded the histochemical staining with combined alcian blue-safranin, according to the method described by Csaba (1990) and modified by Gaje et al. (2007). Antigen retrieval was performed in buffer citrate pH6, using the PTlink module (DakoCytomation, Denmark). The incubation with the primary antibody CD34 (Dako GlostrupDenmark, monoclonal mouse anti-human, clone 1A4, ready to use) for 30 min was followed by visualisation (NovoLink Max Polymer Detection System Leica Biosystems, Newcastle uponTyne, UK). 3,3 diaminobenzidine dihydrochloride served as a chromogen, and hemotoxylin was used for counterstaining. The entire immunohistochemical procedure was developed with DakoAutostainer plus (DakoCytomation, Denmark). The double staining allowed the counting of microvessel (MVD) and mast cell (MCD) density on the same section simultaneously. Microscopic examination was performed with a Nikon-Eclipse 600 microscope. The counting of mast cells and blood vessels was based on the procedure published by Weidner et al. (1995). Briefly, three fields from the tumor area with maximum density of both mast cells and blood vessels were counted for each case. Statistical analysis was performed with SPSS15.0 in order to evaluate the relationship between MCD and MVD;  $p < 0.05$  was considered as significant.

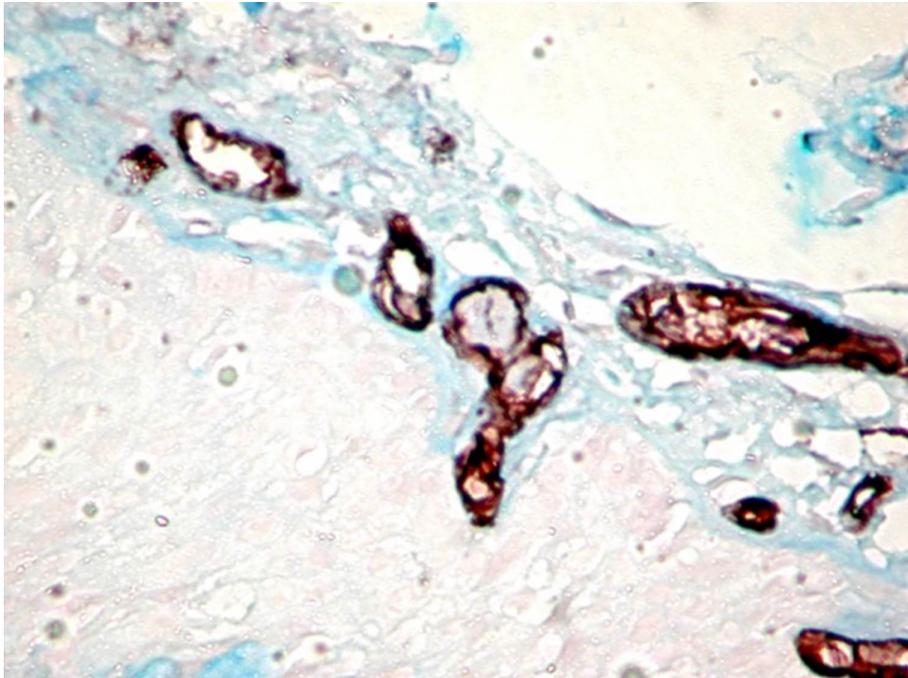
## RESULTS

The diagnosis based on haematoxylin-eosin staining revealed the following types of cancer: adenoid cystic carcinoma (2 cases), malignant papilloma (2 cases) and squamous cell carcinoma (34 cases). According to the grade of the tumor, they were classified as well differentiated laryngeal carcinoma – G1 stage (18 cases), moderately differentiated – G2 stage (12 cases) and poorly differentiated – G3 stage (8 cases).

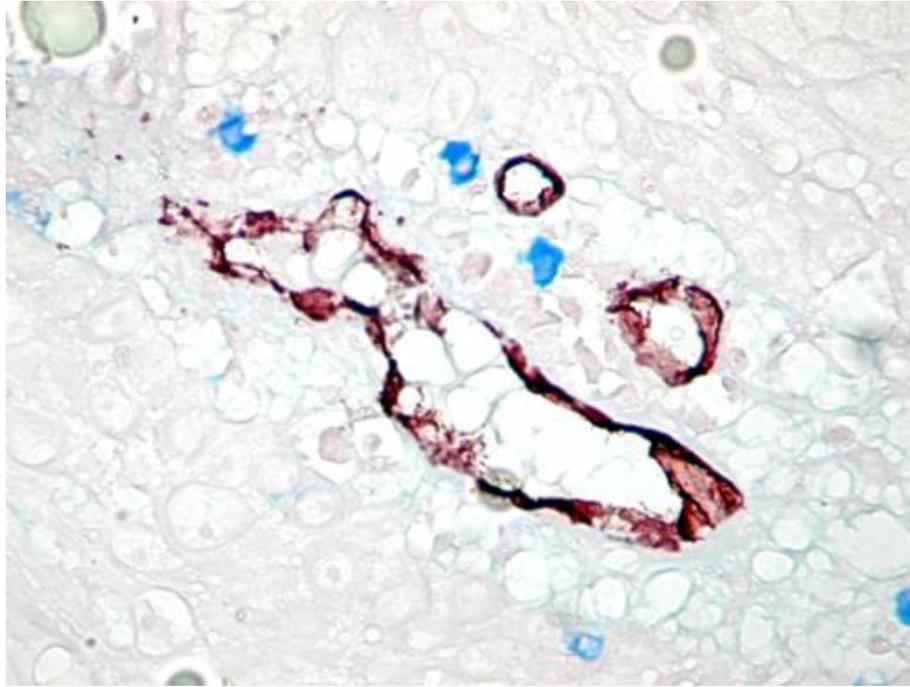
In the cases of adenoid cystic carcinoma (G1 stage), necrosis, keratotic pearls and peritumoral inflammatory infiltrate were absent, but a high number of fibroblasts were found in the dense stroma. Tumor cells had basophilic cytoplasm, uniform nuclei with small nucleoli, rare or absent mitoses.



**Fig. 1.** Alcianophilic mast cells distributed in the vicinity of glands; CD34/ABS staining; x 100 magnification.



**Fig. 2.** Vessels with a large lumen and continuous wall in malignant papilloma; CD34/ABS staining; x 200 magnification



**Fig. 3.** Alcianophilic mast cells with a perivascular distribution in squamous cell carcinoma; G1 stage; CD34/ABS staining; x 200 magnification.

In two cases, squamous cell carcinomas were found, well differentiated (G1 stage), developed on base of papillomatosis. The deep portion presents cellular proliferation and it was composed of cells with compact or moderately discohesive arrangement.

In the cases of keratinized squamous cell carcinoma, for G2 stage, moderately differentiated laryngeal carcinomas, the proliferation of malignant cells and the arrangement in large islands or compact areas was observed. An abundant inflammatory infiltrate was found in the stroma. The tumor cells were large, with vesicular nuclei, large and unequal nucleoli and frequent atypical mitosis. The cytoplasm was moderately acidophilic and degenerated granulocytes were observed among the tumor cells. In some areas, the tumor presented complete keratinization marked by intense acidophilic cytoplasmic keratosis and parakeratotic pearl formation.

Invasive squamous cell carcinoma (G3 stage) was characterized by relatively large necrotic areas, with tumor cells in large placards and islands separated by

connective stroma rich in a predominantly lymphocytic inflammatory infiltrate. The tumor cells varied in size, being mostly polygonal with a large and euchromatic nucleus, large and multiple nucleoli and a weakly acidophilic cytoplasm.

In the cases of adenoid cystic carcinoma, CD34/ABS staining showed the presence of small and immature vessels, without a visible lumen. Mast cells were stained in blue, predominantly distributing close to the glands (Fig. 1). The highest and almost similar values for mast cell and microvessel density were found in this histopathological type.

In the case of malignant papilloma, the vessels presented a large lumen, continuous wall and a value of 15 for the microvascular density (Fig. 2). The mast cell density value was 10; the mast cells stained red, and most of them were in a degranulated state, mainly found in the stroma as large groups and between the epithelial cells as isolated cells.

In the invasive squamous cell carcinoma (G1 stage), in the tumor area vessels of various size, irregular branched, with very thin walls and endothelial cell buds were discerned. When present, the mast cells were located in the immediate vicinity of the vessels (Fig. 3). The values for mast cell density ranged between 8 and 80, and 0 and 28 for microvascular density.

The G2 differentiated grade was characterized by large vessels, with thin walls and irregular shape, associated with severe inflammation in the tumor stroma. The evaluation of microvascular density indicated values between 24 and 80 per microscopic field (x 200). Almost all mast cells present in squamous cell carcinomas, G2 and G3 stage, were stained blue. For the mast cell density of G2 differentiated grade the values ranged between 8 and 21 per microscopic field. The lowest values of mast cell density, between 0 and 5 per microscopic field were observed in the G3 differentiation stage.

A significant correlation between mast cell density and histological grade G1/G2 was found ( $p=0.002$ ). The same aspect was observed between the degrees of differentiation G1, G2 and microvascular density ( $p=0.004$ ). A significant correlation between mast cell density and histopathological type of squamous cell carcinoma was observed ( $p=0.003$ ). The histopathological type of squamous cell carcinoma was not correlated with microvascular density ( $p=0.454$ ).

## DISCUSSION

Several cellular elements of the extracellular matrix, growth factors and specific inhibitors are involved in angiogenesis. At the cellular level, angiogenesis depends on endothelial cells, pericytes, macrophages, fibroblasts and mast cells status.

Mast cells are normal connective tissue residents, and their density varies from one organ to another; they are always well represented in the respiratory tract. A mutual spatial and functional relationship is observed between mast cells (MCs) and endothelial

cells, and the density of MCs correlate with the extent of tumor angiogenesis (Guidolin et al, 2006). Experimental data support the fact that tumour progression is associated with angiogenesis, and a microvessel density (MVD) increase is associated with a higher mast cell density (MCD) (Ranieri et al., 2003).

The relationship between microvessel and mast cell density was extensively studied in different organs. Thus, in a study of the mast cells' effect on tumor angiogenesis in lung cancer, Tomita et al. (2000) established a direct correlation between the number of mast cells and tumor angiogenesis in patients with lung cancer, and this relationship appears to be independent of vascular endothelial growth factor (VEGF) expression. The involvement of tumor microenvironment, and of mast cells in particular in angiogenesis was shown in hematological malignancies, such as non-Hodgkin lymphoma (Duşu et al., 2011).

In a study of the correlation between mast cells and human hepatocellular carcinoma (HCC), Grizzi et al. (2003) stressed that the lack of any significant correlation between MC density and the stage or grade of the neoplastic lesions suggests that there is no causal relationship between MC recruitment and HCC.

In another study on the mast cell profile in the uterine cervix, Naik et al. (2004) compared the mast cell densities in neoplastic and non-neoplastic conditions, revealing an increase in chronic inflammatory processes, while in cancers there was a decrease in number or a total absence of mast cells. The authors established an inverse relationship existed between the mast cell population and the degree of anaplasia.

In the head and neck area, Elpek et al. (2001) showed that microvessels density (MVD) is a reliable prognostic marker of SCC of the esophagus. Moreover, mast cell density (MCD) may have a role in the angiogenesis of these tumors and might be responsible for their aggressive behavior (Gu et al., 2013).

In laryngeal cancer, the expression of extracellular matrix protein 1 was higher in laryngeal carcinoma when compared with other laryngeal lesions and the ECM1 expression in patients with metastasis was significantly higher than in patients without metastasis (Barth et al., 2004).

Of the cellular components of the tumor microenvironment – fibroblasts, carcinoma-associated fibroblasts, endothelial cells, smooth muscle cells, myofibroblasts, pericytes – mast cells are important players in the angiogenesis of laryngeal cancer, their number increases with angiogenesis, according with some authors (Iamaroon et al., 2003; Sawatsubashi et al., 2000; Barth et al., 2004). Opposite results regarding mast cell density in laryngeal carcinoma were found by other authors (Leedy et al., 1994; Oliveira-Neto et al., 2007). One possible explanation for the conflicting results may be the omitted mast cell granule content. This is the reason why in the present study CD34/AAS double staining was chosen for a better correlation between mast cell granules contents, differentiation grade and histological type. In the case of laryngeal adenoid cystic carcinoma and squamous cell carcinoma, mast cells were basophilic. Acidophilic mast cells with safranin-positive cytoplasm were found in the malignant papilloma case.

It was shown that increased angiogenesis was an early event in laryngeal tumor development (Laitakari et al., 2004). The vessels' distribution was related to the degree of differentiation in the squamous cell carcinoma. In our study, we found a significant correlation between microvessel and mast cell density and G1/G2 differentiation grade. These results are contrary to the study that showed that mast cell density was unrelated to the degree of differentiation of the tumor (Parizi et al. 2010).

In oral squamous cell carcinoma the MVD, MCD and the correlation between them significantly increases from normal oral mucosa, dysplasia or OSCC. No significant differences in mast cell and microvessel density between low and high grades of OSCC was found (Mohtasham et al., 2010; Mi-

chailidou et al., 2008). In our study, the squamous cell carcinoma histopathological type significantly correlated with mast cell density, but not with microvascular density.

## CONCLUSIONS

During early stages G1/G2, a significant correlation was found between these differentiation grades and both MCD and MVD. Squamous cell carcinoma histopathological type was significantly correlated with MCD, but not with MVD. Adenoid cystic carcinoma had the highest MCD and MVD, while malignant papilloma had the lowest density values. Our results underline the involvement of alcianophilic and safraninophilic mast cell types in malignant epithelial tumors of larynx.

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