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Immunohistochemical expression of MMP-9 and VEGF in squamous cell carcinoma of the tongue

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Abstract: Squamous cell carcinomas (SCCs) account for approximately 95% of all oral malignant neoplasms and for about 38% of all malignant head and neck tumors, especially affecting the tongue and lips. The aim of this study was to evaluate the immunohistochemical expression of MMP-9 and VEGF in oral SCC according to the occurrence of metastasis. Eighteen cases of tongue SCC without metastases and 17 cases of tongue SCC with metastases were subjected to immunohistochemical methods. High immunohistochemical expression of MMP-9 and VEGF by neoplastic cells and stroma was observed in tongue SCCs at the invasion front. Metastatic tumors tended to express higher levels of MMP-9 and VEGF than non-metastatic tumors, but the difference was not significant ($P > 0.05$). Spearman's correlation test showed no significant correlation between VEGF-immunopositive vessels and metastasis ($P > 0.05$). The present results demonstrate the importance of the expression of MMP-9 and VEGF for the development of SCC of the tongue. However, no significant association was observed between the overexpression of MMP-9 or VEGF and the presence of metastases. (J Ora Sci 54, 105-111, 2012)

Keywords: MMP-9; VEGF; immunohistochemistry; oral squamous cell carcinoma.

Introduction

Squamous cell carcinomas (SCCs) account for approximately 95% of all oral malignant neoplasms and for about 38% of all malignant head and neck tumors (1), especially affecting the tongue and lips (2). The aggressiveness of these tumors depends on numerous factors; however, tongue carcinomas generally exhibit very aggressive biological and clinical behavior (3).

The prognosis of SCC is related to the proliferative activity of the tumor, the degree of differentiation, and the invasion and metastatic potential (4). The last two processes involve multiple steps, including degradation of the basement membrane and extracellular matrix (ECM), alterations in cell adhesiveness, tumor cell motility (5), and angiogenesis (6). The assessment of factors that influence these processes is important for the understanding of tumor behavior and for the development of anticancer therapies.

There is strong evidence that angiogenesis is related not only to tumor growth, but also to the development of metastases (7). Angiogenesis is a dynamic and highly complex process that is regulated by pro- and antiangiogenic molecules (7). In general, increased tumor vascularization and higher expression of proangiogenic factors have been associated with an advanced stage of the tumor and poor prognosis in a variety of human cancers (4).

VEGF is a potent inducer of the formation of blood

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Table 1 Specifications of the antibodies used

Antibody	Manufacturer	Clone	Antigen retrieval	Dilution	Incubation
MMP-9	Novocastra	3C3	Citrate, pH 6.0, 30 min, Pascal	1:20	Overnight
VEGF	Santa Cruz Biotechnology	C-1	Trypsin 0.1% pH 7.9, 37°C, 60 min	1:600	Overnight

vessels and contributes to tumor vascularization and sufficient nutrient supply to sustain its growth. The overexpression of VEGF has been associated with tumor progression and poor prognosis in cases of colorectal, gastric, pancreatic, breast, prostate, lung, kidney, bladder, and ovarian cancers and melanoma (4).

MMP-9 mediates the release of VEGF bound to ECM by the cleavage of extracellular proteins and coordinates the degradation of ECM. Thus, MMP-9 seems to act indirectly on the recruitment of endothelial cells by increasing the release of VEGF into the interstitial medium. This suggests a combined action of MMP-9 and VEGF in the process of angiogenesis (8).

The objective of the present study was to evaluate the immunohistochemical expression of MMP-9 and VEGF in SCC of the tongue in order to determine the presence or absence of a correlation between the expression of these proteins and the occurrence of metastasis. The results are expected to contribute to a better understanding of the biological behavior of tongue SCC and of the roles of MMP-9 and VEGF in the metastatic process of these tumors.

Materials and Methods

The study samples consisted of 35 paraffin-embedded tissue specimens of SCC; incisional biopsies and specimens with inadequate material or extensive areas of necrosis were excluded from the study. Eighteen cases of SCC of the tongue without metastases and 17 cases of SCC of the tongue with metastases were obtained from the files of the Oral Pathology Service. Patients were surgically treated without prior radiotherapy or chemotherapy. The study was approved by the Research Ethics Committee.

Immunohistochemical methods

For immunohistochemistry, 3- μ m thick sections were mounted on glass slides previously prepared with organosilane adhesive (3-aminopropyltrithoxysilane, Sigma Chemical Co., St. Louis, MO, USA) and submitted to the streptavidin-biotin method. The histological sections were deparaffinized in xylene and rehydrated in a decreasing alcohol series. The sections were then submitted to antigen retrieval (Table 1) and

blockage of endogenous peroxidase with 10 volumes of hydrogen peroxide, washed in water, and incubated with Tris-HCl, pH 7.4, for 10 min. Next, the sections were incubated with the primary antibodies (Table 1) diluted in 1% bovine serum albumin/Tris-HCl, pH 7.4. The reactions were developed with 0.03% diaminobenzidine as a chromogen and the slides were counterstained with Mayer's hematoxylin for 10 min. Finally, the sections were dehydrated in alcohol and cleared in xylene for mounting in Permount resin (Fisher Scientific, Fair Lawn, NJ, USA) under a coverslip. Liver and kidney sections were used as positive controls for MMP-9 and VEGF, respectively. Samples were treated as described above, except that the primary antibody was replaced with a solution of bovine serum albumin in phosphate-buffered saline, served as negative controls.

Evaluation of immunostaining and statistical analysis

Immunostaining at the invasion front was evaluated by two examiners at different times under a light microscope. Staining for MMP-9 and VEGF was evaluated in neoplastic and stromal cells. Expression was analyzed semiquantitatively and scored as follows according to the method proposed by Franchi et al. (9) and adapted to this study for statistical analysis: 0, no stained cells; 1, $\leq 25\%$ stained cells; 2, $> 25\%$ and $\leq 50\%$ stained cells; 3, $> 50\%$ and $\leq 75\%$ stained cells; and 4, $> 75\%$ stained cells.

Additionally, the mean number of VEGF-immunopositive vessels was determined based on a method adapted from Maeda et al. (10). Tissue sections were examined by light microscopy at $\times 40$ magnification and five areas showing the highest vascularization were identified subjectively. In these areas, only vessels with a conspicuous lumen were counted at $\times 200$ magnification.

Immunohistochemical staining of MMP-9 and VEGF was evaluated using descriptive and semiquantitative methods. Statistical analysis was performed using the SPSS for Windows software program, version 17.0 (SPSS, Chicago, IL, USA). Comparisons between groups were made using the chi-square test. Spearman's correlation test was used to evaluate the correlation between VEGF-immunopositive vessels and metastasis. A *P* value < 0.05 was considered to indicate statistical significance.

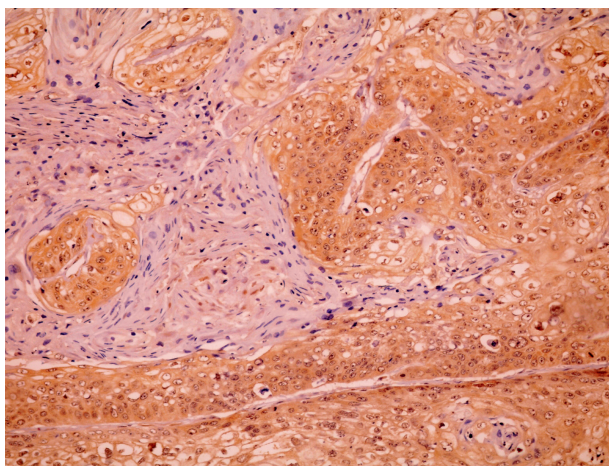


Fig. 1 Squamous cell carcinoma of the tongue showing MMP-9 staining that is more intense in the parenchyma than in the tumor stroma ($\times 200$).

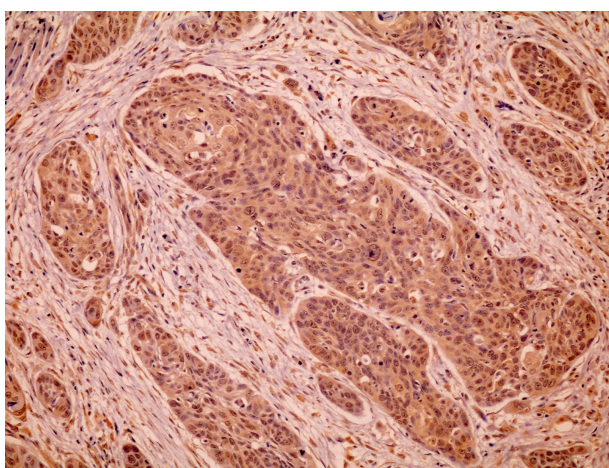


Fig. 2 Non-metastatic squamous cell carcinoma of the tongue showing intense immunorexpression of MMP-9 in the parenchyma ($> 75\%$ positive cells) ($\times 200$).

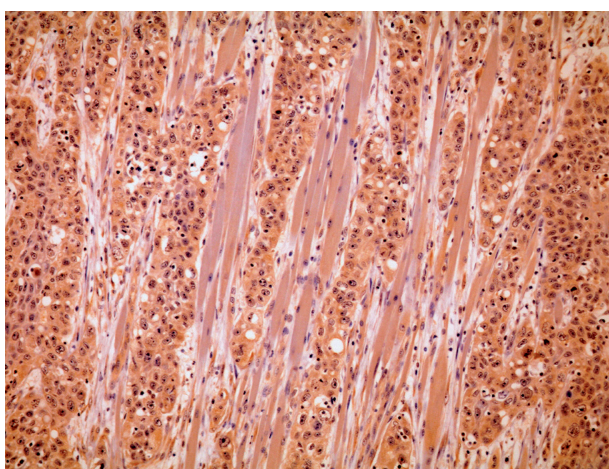


Fig. 3 Metastatic squamous cell carcinoma of the tongue showing intense immunorexpression of MMP-9 in the parenchyma ($> 75\%$ positive cells) ($\times 200$).

Results

At diagnosis, the age of the patients varied between 40 and 91 years (mean age, 64.2 years). There were more men than women, a ratio of 2:1. Metastases were more common among men (58.8%). Microscopically, the SCCs revealed proliferation of epithelial cells arranged in solid sheets, nests, islands, and cords invading the connective tissue. The cells showed cellular and nuclear pleomorphism, hyperchromatic nuclei, and conspicuous nucleoli. An increased nuclear/cytoplasmic ratio, a variable degree of keratinization and even formation of keratin pearls, and an increased frequency of mitotic abnormalities were observed. The tumor stroma consisted of fibrous connective tissue with blood vessels and inflammatory infiltrate, predominantly mononuclear, of various intensities. Lymphovascular, perineural, and perimuscular invasion was seen.

MMP-9 and VEGF were expressed in all specimens. The immunorexpression of these proteins was confirmed by the presence of brown stained cytoplasm in tumor cells, vascular endothelial cells, inflammatory cells, and fibroblasts.

MMP-9 was expressed in tumor and stromal cells at the invasion front in all tongue SCC cases studied. In general, MMP staining was more intense in the parenchyma than in the tumor stroma (Fig. 1). With respect to the immunorexpression of MMP-9 in parenchyma, 27 (77.1%) tongue SCCs were classified with a score of 4 ($> 75\%$ positive cells), including 13 (72.2%) non-metastatic cases (Fig. 2) and 14 (82.4%) metastatic cases (Fig. 3) (Table 2). Metastatic tumors tended to express higher levels of MMP-9 than non-metastatic tumors, but the difference was not significant ($P > 0.05$) (Table 2). Similarly, in the stroma, immunoreactivity to MMP-9 was classified with a score of 4 in most cases (57.1%). Of these, 64.7% were metastatic tongue SCCs and 50% were non-metastatic tumors, with no significant differences between groups ($P > 0.05$) (Table 2).

VEGF was expressed in tumor and stromal cells at the invasion front in all specimens analyzed. With respect to the immunorexpression of VEGF in the parenchyma of tongue SCC, 32 (91.4%) cases were classified with a score of 4 ($> 75\%$ positive cells), including 16 (88.9%) non-metastatic cases (Fig. 4) and 16 (94.1%) metastatic cases (Fig. 5 and Table 2). Similar findings were obtained for the immunorexpression of VEGF in the stroma. Sixteen (88.9%) non-metastatic cases and 16 (94.1%) metastatic cases were classified with a score of 4 ($> 75\%$ positive cells). No significant differences between groups were observed in the two analyses ($P > 0.05$) (Table 2).

The number of VEGF-immunopositive vessels ranged

Table 2 Immunohistochemical expression of MMP-9 and VEGF in metastatic and non-metastatic SCC of the tongue

		Metastatic Scores n (%)					Non-metastatic Scores n (%)					P
		0	1	2	3	4	0	1	2	3	4	
MMP-9	Parenchyma	0 (0%)	2 (11.8%)	0 (0%)	1 (5.9%)	14 (82.4%)	0 (0%)	2 (11.1%)	0 (0%)	3 (16.7%)	13 (72.2%)	0.604
	Stroma	0 (0%)	2 (11.8%)	1 (5.9%)	3 (17.6%)	11 (64.7%)	0 (0%)	1 (5.6%)	4 (22.2%)	4 (22.2%)	9 (50%)	0.484
VEGF	Parenchyma	0 (0%)	0 (0%)	0 (0%)	1 (5.9%)	16 (94.1%)	0 (0%)	0 (0%)	0 (0%)	2 (11.1%)	16 (88.9%)	0.522
	Stroma	0 (0%)	0 (0%)	0 (0%)	1 (5.9%)	16 (94.1%)	0 (0%)	0 (0%)	0 (0%)	2 (11.1%)	16 (88.9%)	0.522

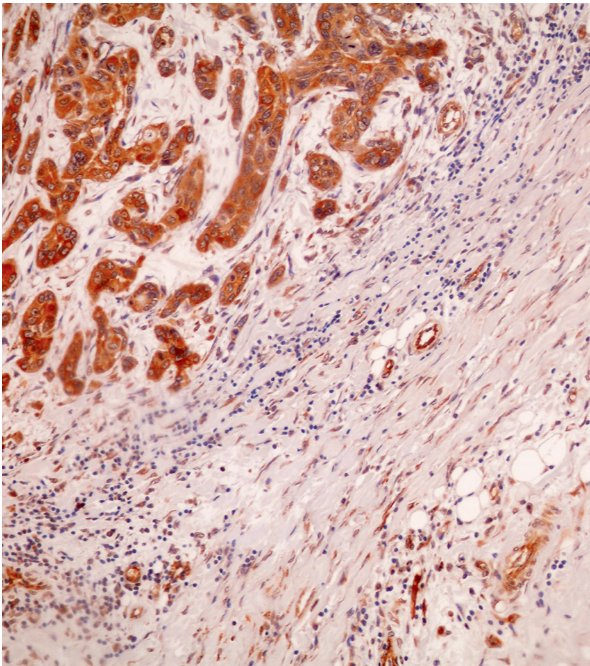


Fig. 4 Non-metastatic squamous cell carcinoma of the tongue showing intense immunoreactivity of VEGF in the parenchyma (> 75% positive cells) ($\times 100$).

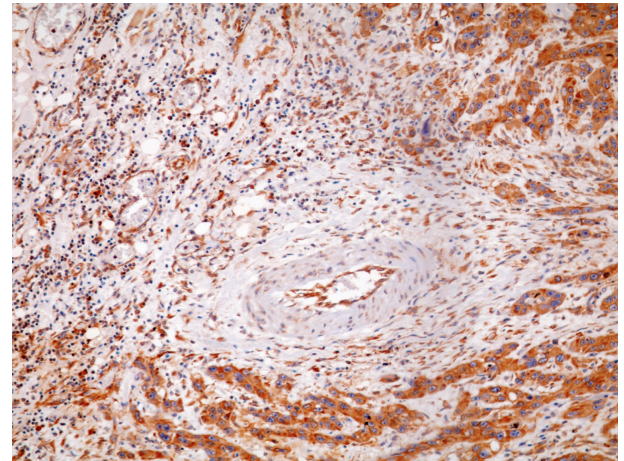


Fig. 5 Metastatic squamous cell carcinoma of the tongue showing intense immunoreactivity of VEGF in the parenchyma (> 75% positive cells) ($\times 100$).

from 4 to 29 vessels (mean: 14.3, SD: 7.3) in non-metastatic tongue SCC and from 5 to 20 (mean: 11.8, SD: 4.6) in metastatic tumors, with no significant differences between groups ($P > 0.05$). The Spearman correlation test showed no significant correlation between VEGF-immunopositive vessels and metastasis ($P > 0.05$).

Discussion

Immunohistochemical analysis of MMP-9 and VEGF at the tumor invasion front demonstrated an overall high expression of these proteins in the cases of tongue SCC studied, suggesting that these molecules play an effective role in the development of these tumors.

The abundant staining for MMP-9 clearly reflects the importance of this enzyme for the invasion of adjacent tissues by the tumor through the destruction of ECM components, especially collagen IV (8). Duffy et al. (11)

and Werner et al. (12) showed that the overexpression of MMP-9 is correlated with an invasive phenotype and metastatic potential of tumor cells.

Several studies have investigated the role of MMP-9 in oral SCC and demonstrated a relationship between the expression of this gelatinase and tumor aggressiveness (13), a fact permitting the use of this MMP as a prognostic marker for therapeutic purposes. Preclinical trials have shown that SCCs express high levels of MMPs *in vivo* and that inhibition of these enzymes *in vitro* and in animal models is associated with invasiveness and metastasis (14). However, other studies were unable to confirm these results (4,9).

The present study showed that tumor and stromal cells of the oral SCC cases studied produced MMP-9. However, expression was higher in the parenchyma. It is believed that these stromal enzymes potentiate the action of MMPs produced by the parenchyma. This fact supports the view of an interaction between neoplastic cells and the adjacent stroma as demonstrated in some experiments (15,16). This strategic interaction permits neoplastic cells to induce stromal cells to produce proteolytic enzymes that act in synergism with tumor enzymes,

thus facilitating the processes of invasion, migration, and metastasis.

The presence of metastasis has been used as a parameter to test the efficiency of possible markers of tumor aggressiveness. Hong et al. (17) observed a strong expression of MMP-2 and MMP-9 in metastatic oral SCCs. Franchi et al. (9) and Katayama et al. (13) reported a significant correlation between the expression of MMP-9 and the occurrence of nodal or distant metastases in head and neck carcinomas. In contrast, other investigators (4,18) observed no significant correlation between the presence of metastasis and the expression of MMP-2 or MMP-9. In the present study, although MMP-9 immunoreactivity tended to be higher in the parenchyma and stroma of metastatic carcinomas, no significant difference was observed.

Nevertheless, Georges et al. (19) emphasized that MMPs promote tumor progression not only through the degradation of ECM but also through the regulation of angiogenesis. Engsig et al. (8) suggested a combined action of MMP-9 and VEGF in this process. In agreement with these findings, in the present study, the expression of MMP-9 in the parenchyma and stroma was accompanied by a similar intensity of VEGF staining, suggesting a possible interaction between these molecules.

Angiogenesis plays an important role in tumor growth and metastasis and some growth factors, such as basic fibroblastic growth factor, interleukin-8, platelet-derived growth factor, and VEGF, are expressed by tumors (20). VEGF is probably one of the most important proteins of the growth factor family in terms of angiogenesis regulation. VEGF is a potent proangiogenic factor and is an essential growth factor for vascular endothelial cells. It is produced by different cells such as vascular smooth muscle, endothelial, and inflammatory cells (21). VEGF mRNA has been detected in numerous tumors, with VEGF immunoreactivity being localized in tumor cells and stromal matrix. VEGF is released into the surrounding stromal matrix and may contribute to tumor growth and metastasis in a paracrine manner through angiogenesis and by increasing vascular permeability. In addition, VEGF induces endothelial cell proliferation, promotes cell migration, and inhibits apoptosis. Dysregulation of VEGF expression contributes to the development of solid tumors by promoting tumor angiogenesis (22) and is associated with increased metastasis (23).

In the present study, overexpression of VEGF was observed in both the parenchyma and the tumor stroma, indicating a higher ability to increase vascular permeability, possibly resulting in increased tumor growth. Overexpression of VEGF has been associated with tumor

progression and poor prognosis in several tumors, such as colorectal, gastric, pancreatic, breast, prostate, lung, kidney, bladder, and ovarian cancers and melanoma (24).

It is well established that oral SCC preferentially spreads through the lymphatic system and that the presence of lymph node metastasis at the time of diagnosis is one of the reasons for treatment failure (25). VEGF does not seem to promote lymphangiogenesis, whereas other members of the VEGF family such as VEGF-C and VEGF-D are responsible for tumor lymphangiogenesis and lymph node metastasis (25). Studies investigating this association have reached different conclusions. Three studies (26,27) reported a statistically significant association between the overexpression of VEGF and the presence of lymph node metastasis, whereas other investigations including the present one did not find such association (4,20,28). Accordingly, this would indicate that the expression of VEGF in oral SCC does not play a determinant role in regional lymph node metastasis.

Furthermore, tumor cells may produce VEGF not only for vessel sprouting, but also to use it as an autocrine growth factor. Some studies have demonstrated the existence of VEGF receptors in oral SCC cells (29,30), suggesting an autocrine role of VEGF. These aspects might explain the lack of a significant association between VEGF expression and angiogenesis. In the present study, we determined the mean number of VEGF-immunopositive vessels to evaluate stromal VEGF immunoreexpression. No significant correlation was observed between the mean number of VEGF-immunopositive vessels and metastasis. Other investigators also failed to show a correlation between VEGF expression and microvessel density in SCC of the head and neck (7,31), whereas a strong correlation was reported in two other studies (30,32). In contrast, Kyzas et al. (25) found no correlation between microvessel density and metastasis.

The present results clearly demonstrate the marked expression of MMP-9 and VEGF in SCC of the tongue. Although this expression was more prominent in parenchymatous cells, it is believed that the tumor stroma is also a determinant factor for tumor progression. Despite the lack of statistical significance, the expression of MMP-9 and VEGF tended to be high in metastatic tongue SCC.

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