

The prognostic value of CXCR4, MMP-2 and MMP-9 in tongue squamous carcinoma

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Abstract

Currently, tongue squamous cancer appears to be more frequent, especially among adults under the age of 45. Approximately 50% of these patients are diagnosed late, with clinically detectable metastases; the five-year survival rate of patients with loco-regional metastases is less than 60%. In order to explain this behavior, many investigations have been conducted in recent years, most of them focusing on identification of potential prognostic and therapeutic markers involved in the pathogenesis of tongue cancers. Our research follows the same trend, which aims to study the prognostic implications of immunohistochemical (IHC) expression of markers C-X-C chemokine receptor type 4 (CXCR4), matrix metalloproteinase (MMP)-2 and MMP-9 in 54 cases of tongue squamous carcinoma. The cases were selected from the archives of the Laboratory of Pathology, Emergency County Hospital, Craiova, Romania, from the 2015–2017 period. They were immunohistochemically processed using the labeled Streptavidin–Biotin (LSAB) enzyme detection technique, and as a method of evaluating reactions, the IHC score developed by Remmele & Stegner. Reactivity for the investigated markers was recorded in both primary tumors, parenchymal and stromal, and in lymph node metastases, and also in normal or dysplastic mucosa adjacent to tumor lesions. The maximum tumor reactivity was recorded for CXCR4, followed by MMP-9 and MMP-2. In addition, all of these markers were expressed stronger in the invasion front and especially in the lymph node metastatic forms. This immunoprofile would suggest their implication in loco-regional invasion and dissemination processes, allowing the selection of the most aggressive forms of tongue squamous carcinoma.

Keywords: tongue, squamous, prognostic, CXCR4, MMP-2, MMP-9.

Introduction

Although the global trend is a decrease of oral squamous cell carcinoma (SCC) incidence, there has been an increase in tongue localization, especially among adults less than 45 years old [1, 2]. This can be explained in part by changing the exposure profile of risk factors associated with classical risk factors (smoking and alcohol consumption) or solitary action of human papillomavirus (HPV) infections (16, 18 and possibly other), other infections with oncogenic viruses, genetic abnormalities and/or as a consequence of exposure to other environmental agents more or less known to have carcinogenic action [3–5]. The most prominent prognostic factors for tongue cancer seem to remain the tumor, node, metastasis (TNM) stage and topographic location. Thus, at the time of diagnosis, at least 50% of patients with tongue cancer have either clinical detectable metastases or undetectable metastases, which greatly reduce the survival rate [6, 7]. According to data provided by the *American Cancer Society* (ACS), the five-year survival rate of patients with local metastases is 78%, decreasing to 63% in patients with regional metastases, and in the case of those with remote metastases to 36% [8]. On the other hand, cases located of the tongue base appear to have the lowest survival rate at five years, respectively 42.6% [9].

Most studies indicate oral tumor genesis as a multi-stage process, in which multiple genes alterations would occur and as a result, a disruption of oncogenes and suppressor genes function. Also, an abnormal increase in growth factor secretion, overexpression of surface receptors, hyperactivity of intracellular signaling pathways and transcription factors, all compete with carcinogenesis [10]. Many of these events are scarcely elucidated, and a lot of research is currently underway on the main prognostic and therapeutic factors involved in the pathogenesis of tongue cancers.

Aim

Our investigation also aims to study the prognostic implications of immunohistochemical (IHC) expression of C-X-C chemokine receptor type 4 (CXCR4), matrix metalloproteinase (MMP)-2 and MMP-9 markers in 54 cases of squamous carcinoma of the tongue.

Materials and Methods

A number of 54 cases of squamous carcinoma localized to the tongue, diagnosed and operated between 2015–2017, were investigated in the Department of Oral and Maxillo-Facial Surgery and in the Department of Surgery, Emergency County Hospital, Craiova, Romania. For the

IHC processing, the corresponding histopathological blocks were used from the archives of the Laboratory of Pathology of the same Hospital.

After reviewing of the histopathological specimens, 4 µm serial sections were made from the selected paraffin blocks, which were applied to electrostatically-charged glass slides. They were subjected to the classic IHC processing protocol using the labeled Streptavidin–Biotin 2 (LSAB2) enzyme detection system and the Dako kit (Redox, Romania – K0675).

Table 1 presents the primary antibodies used in the study along with their main characteristics.

Table 1 – Antibodies used in the study and their main characteristics

Antibody	Clone / Producer	Dilution	Antigen retrieval	External positive control
CXCR4	Rabbit, polyclonal / Thermo Scientific (PA3-305)	1:500	Citrate, pH 6	Squamous carcinoma
MMP-2	Rabbit, polyclonal / Santa Cruz Biotechnology (sc-8835-R)	1:50	0.1 M Citrate, pH 6	Granulation tissue
MMP-9	Mouse, monoclonal 7-11C / Santa Cruz Biotechnology (sc-13520)	1:50	0.1 M Citrate, pH 6	Granulation tissue

CXCR4: C-X-C chemokine receptor type 4; MMP: Matrix metalloproteinase.

The visualization of the reactions was done with 3,3'-Diaminobenzidine (DAB, from Redox, Romania – DAKO, K3468) chromogen and the counterstaining was done with the Mayer's Hematoxylin (from Tunic, Bio-Optica, Romania – M06002). To validate the reactions, we used positive external controls by omitting the primary antibody.

As a method of quantification of IHC reactions, we used the immunoreactivity score (IRS) given by Remmele & Stegner, consisting in examining at ×40 objective at least five tumor areas with maximum tumor reactivity (set at ×10 objective), by determining the percentage of immunoreactive tumor cells multiplied by the intensity of immunoreactions [11]. The percentages of the immunostained tumor cells were: 1 (25% marked cells), 2 (26–49% marked cells), 3 (50–74% marked cells), and 4 (over 75%) and the immunoreactions intensity was rated as: 1 (weak), 2 (moderate), 3 (strong). Finally, the IRS varied between 1–12. The presence of stromal immunoreactivity in this study was evaluated only qualitatively by notifying the presence or absence of reactivity for these markers and identifying their subcellular locations.

The images were captured using the Nikon Eclipse 55i microscope, equipped with a 5-megapixel cooling camera and Image-Pro Plus software. For the statistical analysis, Student's *t*, analysis of variance (ANOVA), χ^2 (chi-square) and Pearson tests were used from the Statistical Package for the Social Sciences (SPSS) 10 software. For statistically testing the probability of association between the different descriptive categories in this study, we made contingency tables with those data, and subsequently used chi-square test.

The results were considered statistically significant when $p < 0.05$. For comparisons on several variables of interest, we used the ANOVA test.

Results

The main clinical and morphological characteristics of the investigated cases are shown in the Table 2. Analyzing the data presented in Table 2, we noticed the prevalence of tongue SCC in people over 60 years (55.56%), male (57.41%), affecting mainly the mobile part of the tongue (66.66%). Histopathologically, moderately differentiated cases (44.44%) predominate and, as well as the pTNM stage, the majority were diagnosed in stage III (37.04%) and stage II, respectively (33.33%).

In the Table 2 are shown the average IRS for each antibody analyzed in relation to the main clinical-morphological variables.

Table 2 – The main clinical-morphological variables of our casuistry and IRS distribution according to these variables

Clinical-morphological variables	No. of cases	[%]	IRS (average ± SD)		
			CXCR4	MMP-2	MMP-9
Age [years]					
<60	24	44.44	1.91± 2.47	0.44± 0.6	1.67± 2.35
>60	30	55.56	2.63± 2.78	0.74± 0.99	2.35± 2.67
Gender					
F	23	42.59	2.02± 2.68	0.41± 0.76	1.96± 2.75
M	31	57.41	2.52± 2.6	0.78± 0.86	2.11± 2.29
Topography					
Mobile portions (including margins)	36	66.66	3.1± 2.75	0.76± 0.87	2.61± 2.57
Fixed portions	18	33.33	1.44± 2.26	0.44± 0.77	1.4± 2.35
Degree of differentiation					
Well differentiated	18	33.33	1.37± 2.24	0.31± 0.51	1.18± 2.02
Moderate differentiated	24	44.44	2.13± 2.77	0.61± 0.96	1.96± 2.78
Low differentiated	12	22.22	1.04± 2.06	0.3± 0.74	0.85± 1.69
pTNM					
I	7	12.96	0.44± 1.26	0.07± 0.26	0.42± 1.42
II	18	33.33	1.34± 2.1	0.31± 0.58	1.18± 1.98
III	20	37.04	1.93± 2.71	0.42± 0.66	1.35± 2.02
IV	9	16.67	0.93± 2.16	0.37± 0.92	1.05± 2.54

IRS: Immunoreactivity score; SD: Standard deviation; CXCR4: C-X-C chemokine receptor type 4; MMP: Matrix metalloproteinase; F: Female; M: Male; pTNM: Pathological tumor, node, metastasis.

IHC study with the CXCR4 antibody

In normal or dysplastic tongue mucosa, the reactivity for CXCR4 was present predominantly in the intermediate layer cells with mainly cytoplasmic and less membranous

immunostaining pattern (Figure 1A). Reactivity was also noted in the associated inflammatory infiltrates, the striated muscle fibers, the epithelium of the extralobular excretory ducts of the minor salivary glands, and the endothelial cells of the blood vessels in the chorion or tumor stroma.

In tumor tissue, we recorded reactivity for CXCR4 in all our investigated cases (100%), but IRS ranged from 1 to 9, the medium value of IRS was 4.54 ± 1.9 . The maximum reactivity (IRS=9) was observed in two cases of G1 and G2 tongue SCCs, developed at the level of the mobile tongue, in a woman older than 60 years and in the stage III pTNM, respectively in a man over

60 years of age and stage IV pTNM. The tumor reactivity pattern was predominantly cytoplasmic and membranous (Figure 1B). We did not notice nuclear reactivity in the tumor cells. Regarding the degree of differentiation, the maximum reactivity was recorded in the moderate and well-differentiated variant; the cells with spinous morphology had the highest reactivity (Figure 1, C and D). In addition, we noticed a slightly higher reactivity in the invasion front, compared to the superficial area of the tumors (Figure 1E). Reactivity for CXCR4 was more evident in lymph node metastatic forms, where reactivity was more evident especially in squamous areas (Figure 1F).

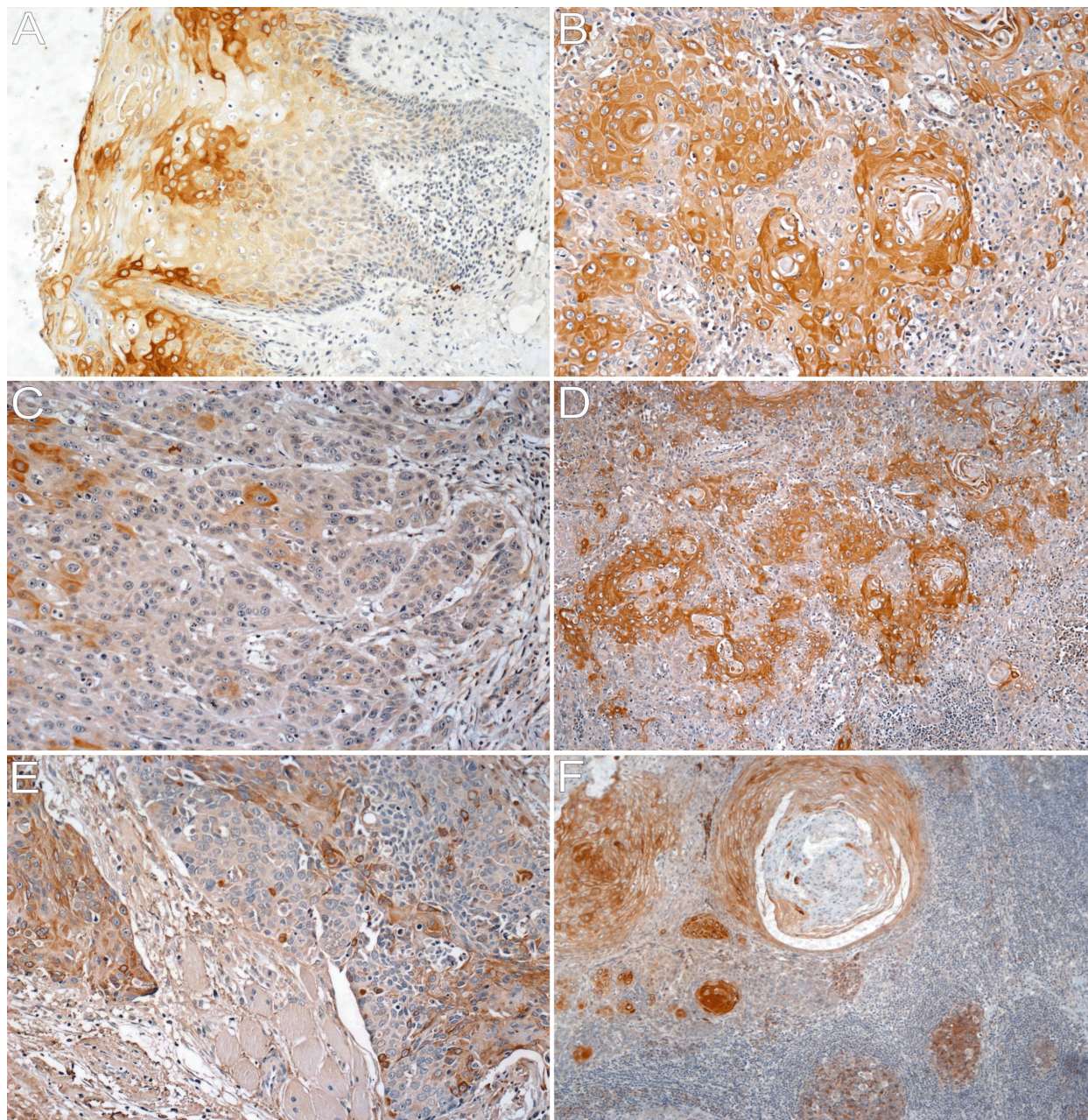


Figure 1 – Tongue SCC: (A) The CXCR4 membranar and cytoplasmic reactivity of the basal and parabasal layer cells from the adjacent tongue tumor epithelium; (B) The CXCR4 predominant cytoplasmic reactivity of tumor cells; (C and D) The CXCR4 cytoplasmic reactivity of tumor cells, in a moderate-differentiated SCC, inside tumor versus invasion front; (E) The CXCR4 cytoplasmic reactivity of tumor cells, from invasive front of moderate differentiated SCC; (F) The CXCR4 predominant cytoplasmic reactivity of tumor cells with squamous cell morphology from the tongue SCC lymph node metastases. Anti-CXCR4 antibody immunostaining: (A and D) $\times 40$; (B and C) $\times 200$; (E and F) $\times 100$. SCC: Squamous cell carcinoma; CXCR4: C-X-C chemokine receptor type 4.

IHC study with MMP-2 antibody

At the level of the tongue epithelium, adjacent to neoplastic lesions, the reactivity for MMP-2 was present in almost its entire thickness, the reaction pattern was predominant at the membrane level and, especially, in the intermediate layer cells. The dysplastic epithelium also showed cytoplasmic responsiveness in the atypical basal cells within the lesion (Figure 2A). Cytoplasmic reactivity for MMP-2 has also been noted in endothelial cells of blood vessels, striated muscle fibers, stromal fibroblasts, macrophages, and epithelium of small salivary gland excretory channels.

Tumor MMP-2 reactivity was recorded in 46 (85.18%) cases, the IRS average being 1.18 ± 1.41 , with values ranging from 0 to 4 (recorded in a female over 60 years, in the

mobile portion of the tongue, with G2 differentiation degree and in stage IV pTNM). The pattern of MMP-2 tumor reactivity was heterogeneous, ranging from membrane pattern, present especially in well-differentiated forms (Figure 2B), to a cytoplasmic visible one, especially in moderately differentiated forms (Figure 2C) and respectively to a nuclear present only in low differentiated tumor forms (Figure 2D). We did not notice differences in tumor reactivity between the invasion front (Figure 2E) and the superficial regions of the tumors.

The MMP-2 tumor reactivity was much more pronounced in tumors with lymph node metastases compared to non-metastatic tumors. Reactivity in metastases was predominantly membranous and less cytoplasmic (Figure 2F).

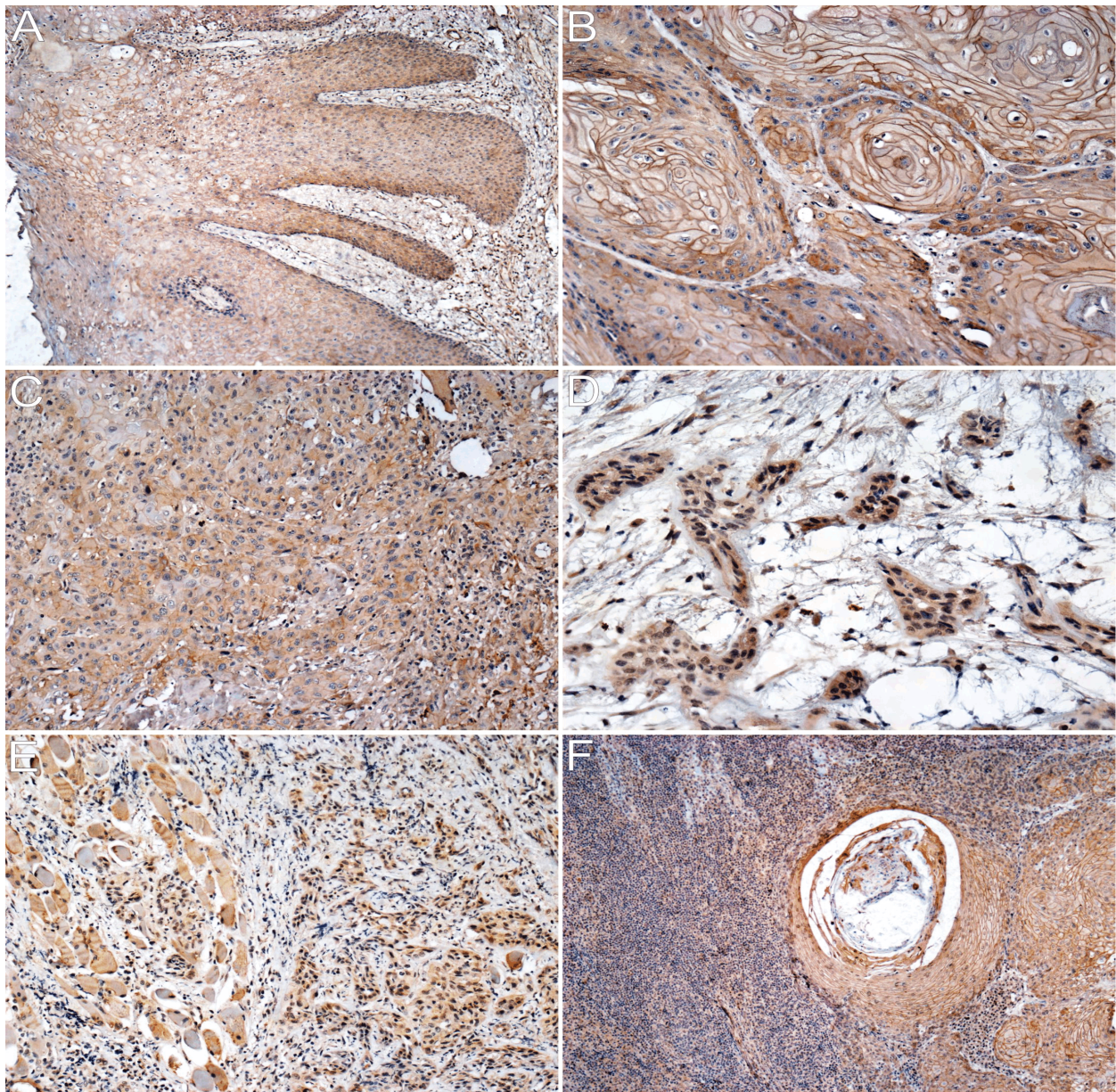


Figure 2 – Tongue SCC: (A) The MMP-2 membranous reactivity of the intermediate layer cells from adjacent tongue SCC epithelium; (B) The predominant MMP-2 membranous reactivity of tumor cells in a well-differentiated tongue SCC; (C) Predominant cytoplasmic reactivity of tumor cells to MMP-2, in a moderately differentiated SCC; (D) Cytoplasmic and nuclear reactivity of tumor cells to MMP-2, in a poorly differentiated SCC; (E) Cytoplasmic and nuclear reactivity of tumor cells to MMP-2, from invasive front of moderately differentiated SCC; (F) Membranous and cytoplasmic MMP-2 reactivity especially of the tumor cells with squamous morphology from the tongue SCC lymph node metastases. Anti-MMP-2 antibody immunostaining: (A and F) $\times 100$; (B–E) $\times 200$. SCC: Squamous cell carcinoma; MMP-2: Matrix metalloproteinase-2.

IHC study with the MMP-9 antibody

In the tongue epithelium, adjacent to neoplastic lesions, the reactivity for MMP-9 was present in the intermediate and superficial layer, the pattern of the reaction being membranous and cytoplasmic (Figure 3A). The cytoplasmic pattern of the reaction was more evident in the tumor-associated hyperplastic lesions. In addition, the cytoplasmic reactivity for MMP-9 has been highlighted in endothelial cells of blood vessels, stromal fibroblasts, inflammatory cells, striated muscle fibers, and epithelial salivary glandular channels.

At tumor level, the reactivity was higher compared

to that recorded for MMP-2, the IRS average for the investigated casuistry being 4.02 ± 2.16 . The IRS varied between 0 and 9. Tumor reactivity was higher in moderate and poorly differentiated forms compared to well-differentiated ones, with cytoplasmic pattern prevailing in moderate cases and the membranous pattern in the poorly differentiated cases (Figure 3, B–D). Also, a higher tumor reactivity was noted especially at the invasion front, prevailing in moderate and low differentiated forms (Figure 3E). Reactivity also occurred in the lymph node metastases (Figure 3F), the reactivity seems to be greater in the metastatic primitive tumors.

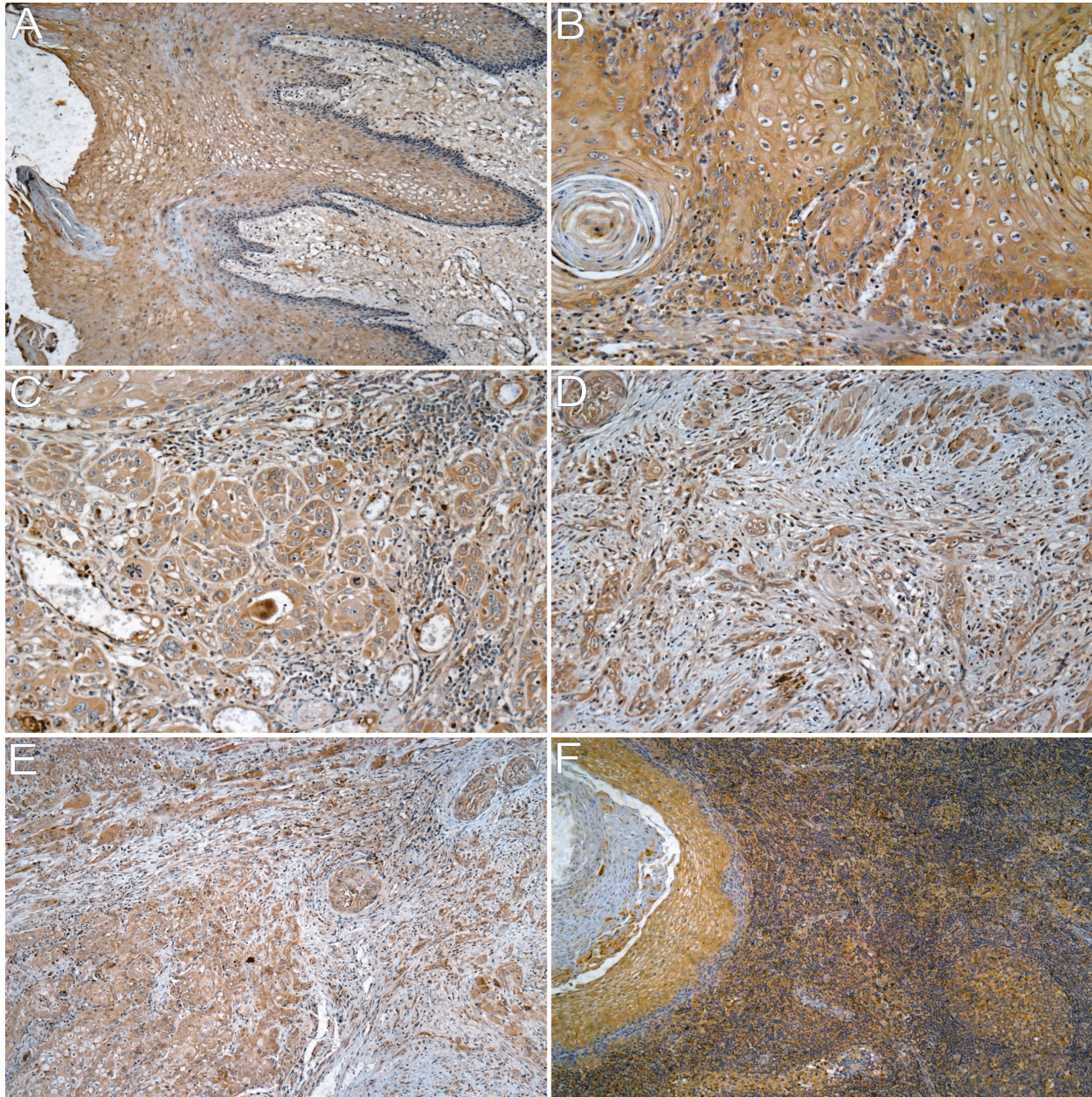


Figure 3 – Tongue SCC: (A) The MMP-9 membranous and cytoplasmic reactivity of the intermediate layer cells from adjacent tongue SCC epithelium; (B) The MMP-9 predominantly membranous and cytoplasmic reactivity of tumor cells, in a well-differentiated SCC; (C) Predominant cytoplasmic reactivity of tumor cells to MMP-9, in a moderately differentiated SCC; (D) Cytoplasmic reactivity of tumor cells to MMP-9, in a poorly differentiated SCC; (E) Cytoplasmic reactivity of tumor cells to MMP-9, from the invasion front of moderately differentiated SCC; (F) The MMP-9 membranous and cytoplasmic reactivity of tumor cells with squamous morphology from the tongue SCC lymph node metastases. Anti-MMP-9 antibody immunostaining: (A, C and F) $\times 100$; (B, D and E) $\times 200$. SCC: Squamous cell carcinoma; MMP-9: Matrix metalloproteinase-9.

Statistical study on reactivity for the three markers

Statistically comparing the IRS obtained for CXCR4 versus MMP-2, we noticed the existence of a poor direct correlation ($r=0.356$, $p<0.05$) between these two markers (Figure 4). Instead, we found a moderate direct correlation between CXCR4 and MMP-9 ($r=0.65$, $p<0.05$) (Figure 5) and between MMP-2 and MMP-9 ($r=0.685$, $p<0.05$) (Figure 6).

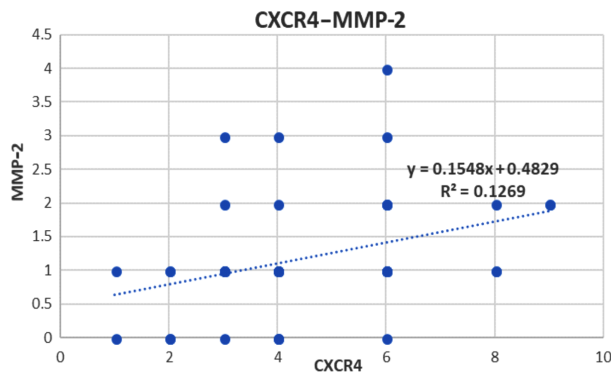


Figure 4 – Statistical analysis of the obtained IRS. A weak direct correlation between CXCR4 and MMP-2 ($r=0.356$, $p<0.05$). IRS: Immunoreactivity score; CXCR4: C-X-C chemokine receptor type 4; MMP-2: Matrix metalloproteinase-2.

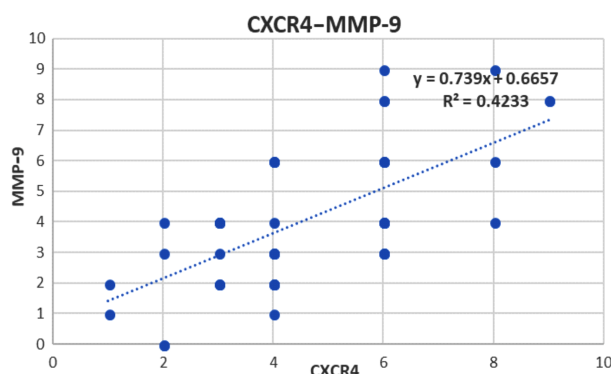


Figure 5 – Statistical analysis of the obtained IRS. Moderated direct correlation between CXCR4 and MMP-9 ($r=0.65$, $p<0.05$). IRS: Immunoreactivity score; CXCR4: C-X-C chemokine receptor type 4; MMP-9: Matrix metalloproteinase-9.

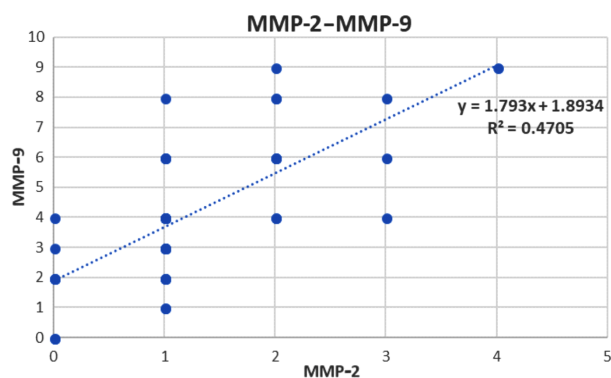


Figure 6 – Statistical analysis of the obtained IRS. A moderate direct correlation between MMP-2 and MMP-9 ($r=0.685$, $p<0.05$). IRS: Immunoreactivity score; MMP-2: Matrix metalloproteinase-2; MMP-9: Matrix metalloproteinase-9.

Statistically analyzing the expression of the three markers in relation to the main morphoclinical variables followed in the study, we noticed a higher expression tendency for each of the three markers, especially in those less than 60 years of age compared to those over 60, for MMP-2 the differences were statistically significant ($p<0.05$). For the pTNM parameter, the ANOVA test revealed significant differences between the four stages only for MMP-2 [$F(3.5)=2.79$, $p<0.001$] and MMP-9 [$F(3.5)=5.17$, $p<0.01$]. In contrast, regarding the tumor-differentiation parameter, the ANOVA test did not reveal statistically significant differences for any of the investigated markers.

Discussions

Tongue cancer has a particular place in oral cancers, considering epidemiology, prognosis and survival. A series of studies show that in tongue cancer at least 50% of the diagnosed cases already present metastasis [6], lymph node disseminations being considered the most important prognostic factor [7]. Moreover, cases with cancers at the base of the tongue, at the time of diagnosis, develops at least 31.4% of contralateral lymph node metastases [6], and the survival rate of such patients is less than 43% [9]. The process of oral carcinogenesis is complex and multistage, following a sequence of events, from normal epithelium is reaching to dysplasia and finally an invasive carcinoma develops. In order to elucidate the molecular profile of oral cancer, in recent decades, a series of genomic and proteomic studies have been carried out, attempting to identify the genetic alterations occurring in oncogenes and other tumor suppressor genes, to determine the degree of involvement of genomic instability and epigenetic changes and to establish a profile of gene expressions that take place during oral oncogenesis [12]. Behind the aggression of tongue cancers is extracellular matrix (ECM) degradation, due to the MMPs secreted by both tumor cells and some of the associated stromal tumor cells [13]. In addition, CXCR4 chemokines have been shown to regulate the secretion of these enzymes, and there have been numerous studies that have shown that tumor expression levels of CXCR4, MMP-2, MMP-9 and MMP-13 in oral squamous carcinomas are coupled [13–15]. In this regard, our study also attempted to investigate the correlations between the expression levels of CXCR4, MMP-2 and MMP-9 in tongue squamous carcinomas, in relation to their main clinical and morphological characteristics and also to investigate their possible prognostic role in the evaluation to such patients.

Chemokines represent a class of small cytokine-like proteins that can bind and activate the family of seven transmembrane receptors coupled to G protein (chemokine receptors) [16]. These chemokines are expressed by a series of tumors and play important roles in initiating mitosis, modulating apoptosis, survival and angiogenesis [17]. The interaction between stromal cell-derived factor-1 (SDF-1) and the CXCR4 chemokine receptor has been shown to play a major role in tumorigenesis, proliferation, metastasis and angiogenesis in a number of human tumors, such as: pulmonary cancer [18], malignant melanoma [19], esophageal cancer [20], ovarian cancer [21], glioblastoma

[22], cholangiocarcinoma [23], and basal cell carcinoma [24]. In oral squamous cancers, CXCR4 has been shown to promote the migration and invasion of cancer cells by regulating the expression of MMP-9 and MMP-13, most likely *via* the activation of the extracellular signal-regulated kinase (ERK) signaling pathway [13].

In our study, we recorded the presence of an immunoreactivity in the intermediate layer of the normal or dysplastic epithelium adjacent to the tumors, as well as in the chorion and tumor stroma at the level of inflammatory cells, vascular endothelial cells, striated muscle fibers and in the epithelium of the extralobular excretory ducts of the minor salivary glands. At tumor level, the reactivity for CXCR4 was superior to the MMP-2 and MMP-9 markers. The registered IRSs varied between 1 and 9. Relative to the degree of differentiation, IRSs were higher in moderate and low differentiated forms, tumor cells with spinous morphology having the highest reactivity. The immunoreactivity pattern was a predominant cytoplasmic and membranous one. In addition, the reactivity appears to be greater at the invasion front and especially in the metastatic forms, the reactivity being also noted in the lymph node metastases.

Literature studies indicate variations in CXCR4 expression in oral squamous carcinomas in percentages ranging from 28.6% to 100% [15, 25–27], differences that can be explained by the use of various expression quantification systems for this marker and/or the use of different CXCR4 clones [28]. In the study undertaken by Xia *et al.*, expression of C-X-C motif chemokine ligand 12 (CXCL12)/CXCR4 was present in both tumor specimens and premalignant oral lesions, suggesting that the CXCL12/CXCR4 axis would play important roles in the premalignant stages of the oral mucosa, contributing to the progression of carcinomas within this localization [29]. A series of studies indicated a direct correlation between CXCR4 expression in primary tumors and the expression of this marker in lymph node metastases [15, 28, 30, 31]. Thus, activation of the CXCR4 receptor would play a major role in the lymph node metastasis of oral squamous carcinomas, the receptor being sensitive to CXCL12 chemoattraction secreted in distant primary tumor assays, targeting tissues for metastatic carcinoma cells expressing high levels of the CXCR4 receptor [15, 25, 30, 32]. In addition, there has been evidence of correlations between the level of CXCR4 tumor expression and the tumor stage (levels being higher in the most advanced stages) [30, 31], respectively correlations with perineal invasion and vascular invasion [30]. On the other hand, carcinoma cell motility, tumor invasion and metastasis in oral squamous carcinomas can be explained by the involvement of the CXCR4 receptor in regulating MMP-9 and MMP-13 expression, as well as by promoting the epithelial–mesenchymal transition (EMT) process [13, 33, 34], demonstrated by correlation of expression of this receptor with vimentin expression level in oral primary tumors [30]. At the same time, literature data indicated that this receptor may be considered as an independent prognostic marker for patients with oral squamous carcinomas [28, 35]. In this regard, a study of SCCs developed at the level of the mobile portion of the tongue showed that patients with tumors that expressed a high level of CXCR4 had a more limited prognosis [30].

MMP-2 is a type IV collagenase of 72 kDa molecular weight, also known as gelatinase A [36], which is involved in the degradation of the ECM under both physiological conditions (embryonic development, endometrial cycle, reproduction, wound healing, bone remodeling) and in various pathological conditions (arthritis, invasion and cancer metastasis) [37]. MMP-2 being involved in the degradation of the collagen IV from the basal membrane component, has a major role in the cancer metastasis process [38]. In addition, invadopodia structures involved in the process of tumor invasion also concentrate the MMPs, including MMP-2, and the degradation products of these enzymes promote the formation of these pro-invasive structures [39]. Also, MMP-2, as other MMPs, determine the proteolytic activation of transforming growth factor-beta (TGF- β), a factor that has been shown to promote EMT process, that has a major role in cancer metastasis [40].

In our study, we documented the presence of MMP-2 immunoreactivity in the normal, hyperplastic or dysplastic epithelium adjacent to tumor lesions, with a particular membranous reaction pattern evident in the intermediate layer cells. A cytoplasmic reactivity was noted in the dysplastic areas. In addition, reactivity for MMP-2 was also noted in the endothelial cells of blood vessels, in the striated muscle fibers, stromal fibroblasts, macrophages and epithelium of the small salivary gland excretory channels. At the tumor level, the reactivity for MMP-2 was inferior to that for CXCR4 and MMP-9. IRS scores for MMP-2 varied between 0 and 4. We did not notice IRS differences of reactivity depending on the degree of differentiation, but differences related to the preponderant pattern of reagents were present. Thus, we recorded preponderant membranous reactivity in the differentiated forms, one of the cytoplasmic types at the level of the moderate forms and a pattern of nuclear reactivity for the poorly differentiated forms. Lymph node metastatic forms appear to be much more reactive than non-metastatic, with metastases accounting for somewhat lower reactivity compared to primary tumors.

Literature studies indicate a higher concentration of MMP-2 (both latent and active) in squamous carcinoma of the head and neck, compared to adjacent normal tissues [41, 42]. Furthermore, it appears that the active form of MMP-2 is in double concentration compared to MMP-9, in the oral squamous carcinoma [41]. Most authors did not find statistically significant correlations between MMP-2 levels and different clinical-morphological parameters, including: gender, tumor stages, nuclear grading, tumor differentiation, smoking exposure [41, 43, 44]. However, most studies indicate a positive correlation between MMP-2 expression and lymph node metastasis and prognosis of patients with squamous carcinoma of the head and neck [41, 45–48]. Pu *et al.* showed that elevated levels of MMP-2 and vascular endothelial growth factor-C (VEGF-C) expression in both primary tumors and corresponding lymph node metastases correlated statistically significantly with the general survival rate of patients with oral carcinomas [48]. However, Mishev *et al.* believes that the level of MMP-2 expression in primary tumors should not be considered as a reliable predictive marker of tumor invasiveness in oral squamous carcinomas [43], while

Katayama *et al.* have failed to establish a correlation between MMP-2 tumor expression and metastatic potential, or prognosis in oral squamous carcinomas [49].

Strictly related to tongue squamous carcinomas, MMP-2 expression level was much higher in tumor tissue compared to normal tongue mucosa or dysplastic lesions in this localization [50]. MMP-2 was expressed not only in tumor cells but also at stromal level, in macrophages and vascular endothelial cells. Thus, the secretion from the MMP tumor stroma is equally important, since their stromal expression level correlated with lymph node metastases and with a worse prognosis [50]. Moreover, several studies of tongue squamous carcinomas have shown that the level of MMP-2 expression in these tumors can be used as a prognostic factor [50–52].

MMP-9, also known as type IV collagenase or gelatinase B, is a matrixin belonging to the large family of dependent zinc metalloproteinases, enzymes involved in the ECM degradation in both physiological processes (embryonic development, reproduction, angiogenesis, bone development, wound healing, cell migration, learning and memory processes) and in pathological processes (arthritis, intracerebral hemorrhage and metastasis) [53, 54]. MMP-9 has been shown to play a major role in neovascularization by proteolytic degradation of basal membrane blood vessels and release of the active form of VEGF [55].

The vast majority of authors note elevated levels of MMP-9 expression in squamous carcinoma of the head and neck, including at oral level, but the prognostic significance of this increased expression remains a contradictory subject. Thus, Guttman *et al.* could not establish a correlation between MMP-9 tumor expression and primary tumor size or laterocervical lymph node metastasis in laryngeal cancers [56]. On the other hand, Katayama *et al.* reported a correlation between the MMP-9 expression rate and the loco-regional lymph node metastasis rate and/or distant metastasis, as well as a poor prognosis [49]. Also, de Vicente *et al.* showed that MMP-9 expression did not correlate with clinical variables, namely tumor stage and relapse rate [57], while Ikebe *et al.* have noted that expression of MMP-2 and MMP-9 has been associated with invasiveness but not with metastatic potential in oral squamous carcinomas [58]. On the other hand, Riedel *et al.* have not found a correlation between MMP-9 expression and T-stage and N-tumor, respectively, but correlated with a worse prognosis in patients with squamous carcinoma of the head and neck [59] while O-Charoenrat *et al.* showed that increased MMP-9 messenger ribonucleic acid (mRNA) levels correlated with advanced T and N stages in head and neck cancers [60]. Kato *et al.* found an increased expression of total MMP-9, its active form was much less expressed compared to MMP-2, and the latter correlated with advanced disease stages [47]. All these contradictory data come to support the idea that MMP-9 is not the only factor involved in the tumor-invasive process of the head and neck and that it can play fluctuating roles in this process [61].

During our study, we observed reactivity for MMP-9 at normal or dysplastic epithelium almost similar to that of MMP-2, but with a higher and obvious intensity at the intermediate and superficial layer. Reactivity for

MMP-9 was also noted in the cytoplasm of endothelial cells of blood vessels, stromal fibroblasts, inflammatory cells, striated muscle fibers, and epithelial salivary gland ducts. At tumor level, the immunoreactivity for MMP-9 was superior to that of MMP-2, IRSs ranging between 0 and 9. Moderate and low differentiated forms were associated with the highest reactivity, the pattern being predominantly cytoplasmic. Tumor reactivity appeared to be higher at the invasion front, especially in low differentiated forms and particularly in metastatic forms compared to non-metastasizing. Reactivity for MMP-9 at the level of lymph node metastases was less comparing with primary tumors.

Statistically, we recorded the existence of moderate direct correlations between CXCR4 and MMP-9, respectively MMP-2 and MMP-9, and between CXCR4 and MMP-2, we observed a poor direct correlation. In addition, significant differences were noted for all three markers analyzed with age and the highest IRS being obtained in individuals aged up to 60 years. At the same time, for the MMP-2 and MMP-9 markers the only statistically significant differences were found for the four-stage pTNM. For the rest of the investigated morpho-clinical parameters we did not observe the existence of statistically significant differences.

Conclusions

Reactivity for the three investigated markers was present in both in parenchyma and tumor stroma, but also in normal mucosa, or dysplasia adjacent to tumor lesions. The biggest tumor reactivity was recorded for CXCR4, followed by MMP-9 and MMP-2. Lymph node metastatic forms have the highest reactivity, suggesting the involvement of these markers in locoregional lymph node dissemination of tongue squamous carcinomas. Tumor reactivity was also higher at the invasion front, suggesting their involvement in invasiveness. Therefore, the three investigated markers can be used as prognostic markers by selecting cases with the most severe prognosis.

Conflict of interests

The authors declare that they have no conflict of interests.

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