Immunohistochemical Evaluation of MMP-2, MMP-9 and CD31/ Microvascular Density in Squamous Cell Carcinomas of the Floor of the Mouth

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The aim of this study was to evaluate the immunoexpression of MMP-2, MMP-9 and CD31/microvascular density in squamous cell carcinomas of the floor of the mouth and to correlate the results with demographic, survival, clinical (TNM staging) and histopathological variables (tumor grade, perineural invasion, embolization and bone invasion). Data from medical records and diagnoses of 41 patients were reviewed. Histological sections were subjected to immunostaining using primary antibodies for human MMP-2, MMP-9 and CD31 and streptavidin-biotin-immunoperoxidase system. Histomorphometric analyses quantified positivity for MMPs (20 fields *per* slide, 100 points grade, ×200) and for CD31 (microvessels <50 μm in the area of the highest vascularization, 5 fields per slide, 100 points grade, ×400). Statistical design was composed by non-parametric Mann-Whitney U test (investigating the association between numerical variables and immunostainings), chi-square frequency test (in contingency tables), Fisher's exact test (when at least one expected frequency was less than 5 in 2x2 tables), Kaplan-Meier method (estimated probabilities of overall survival) and logrank test (comparison of survival curves), all with a significance level of 5%. There was a statistically significant correlation between immunostaining for MMP-2 and lymph node metastasis. Factors associated negatively with survival were N stage, histopathological grade, perineural invasion and immunostaining for MMP-9. There was no significant association between immunoexpression of CD31 and the other variables. The intensity of immunostaining for MMP-2 can be indicative of metastasis in lymph nodes and for MMP-9 of a lower probability of survival.

Introduction

Squamous cell carcinoma (SCC) of the head and neck may be classified by anatomic sites such as the maxilla, oropharynx, larynx and hypopharynx. Although the recommendations for treatment had been developed by tumor site, tumor-node-metastasis (TNM) staging and histological diagnosis, they do not always predict clinical results precisely (1). Despite the slow decline in its incidence (about 4% since 1980), head and neck SCC remains a clinical challenge. In the Brazilian population, the floor of the mouth represents the second most prevalent anatomic site in oral SCC after the tongue (2).

Tumor invasion is a dynamic, complex process with several stages, which involves the displacement of malignant cells from their point of origin, traversing the extracellular matrix and basal membrane and invasion of blood and lymphatic vessels. Proteolysis of the components of the basement membrane and extracellular matrix are essential steps in tissue invasion, therefore specific proteases are required for the degradation of collagen, laminin and fibronectin. For this reason, matrix metalloproteinases (MMPs), which are zinc-dependent proteases, have been studied extensively (3).

A general concept is that increased angiogenesis leads to a greater ability to invade and produce metastases. The precise role of microvascular density (MVD)/CD31+ cells in the prognosis of SCC is yet to be fully established. Some authors believe that a greater MVD leads to a poorer prognosis (4) whereas others believe the opposite (5). In addition, the majority of studies in this field do not make a distinction between different head and neck tumor sites that can display heterogeneous vascular networks (4,6).

In this way, besides showing their importance in the knowledge of tumor pathogenesis, MMPs and angiogenesis could contribute as potential biomarkers in diagnosis and prognosis of oral cancer. For this reason, this study aimed to evaluate the MMP-2, MMP-9 and CD31/MVD immunoexpression in SCC of the floor of the mouth, correlating microscopic parameters with tumor aggressiveness or patient survival.

Material and Methods
Patients

This study was approved by the institutional Ethics Committee (registration protocol #632/04 on January 28th 2004 at A.C. Camargo Cancer Hospital and #177/2004 on

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March 8th 2005 at Bauru Dental School, University of São Paulo).

The eligibility requirements for the 41 individuals participating in this study carried out at the A.C. Camargo Cancer Hospital - Antônio Prudente Foundation (São Paulo, SP, Brazil) were as follows: i) medical records registered with histological diagnosis of primitive malignant neoplasm in floor of the mouth between 1970 and 1993; ii) patient adherence to the treatment with curative purpose (excluding isolated antineoplastic chemotherapy) until its termination; and iii) availability of formalin-fixed and paraffin-embedded human carcinoma samples for immunohistochemical analysis.

Demographic, survival, clinical (TNM staging) and histopathological variables were also reviewed.

Demographic and Survival Variables

Medical records provided the data for demographic analysis (gender, race, habits, age of diagnosis), as well as for the evaluation of overall survival of the patients (lifetime after diagnosis/treatment and duration of follow-up). The last evaluation of the medical records was performed on November 2007.

TNM Staging

Neoplasm staging described in medical records followed the TNM system according to International Union Against Cancer (U.I.C.C.), where: T (Tx - primary tumor could not be identified, T0 - no evidence of primary tumor, Tis - carcinoma *in situ*; T1 - tumor extension <2 cm; T2 - tumor extension >2 cm and <4 cm; T3 - tumor extension >4 cm and T4 - tumor invading adjacent structures), N (Nx - lymph node could not be identified; N0 - no evidence of lymph node involvement; N1 - single ipsilateral lymph node involvement <3 cm; N2a - single ipsilateral lymph node involvement >3 cm and <6 cm; N2b - multiple ipsilateral lymph nodes involvement <6 cm; N2c - bilateral lymph nodes involvement <6 cm; N3 - lymph nodes involvement >6 cm) and M (Mx - distant metastasis could not be identified; M0 - no evidence of distant metastasis; M1 - presence of distant metastasis).

The clustering of cases in clinical stages obeyed the following organization: Stage 0 (Tis N0 M0), Stage I (T1 N0 M0), Stage II (T2 N0 M0), Stage III (T3 N0 M0 or T1-T2-T3 N1 M0), Stage IV (T4 N2-N3 M1).

Histopathological Variables

From the corresponding paraffin blocks, 5-μm-thick sections were prepared, stained with hematoxylin and eosin and examined by a pathologist experienced in this field. Detailed description of the samples included evaluation of perineural invasion, presence of vascular tumor emboli and histological tumor grading (well differentiated, moderately differentiated and poorly differentiated carcinomas).

Immunohistochemical Procedures

Histological sections were mounted on silanized slides with poly-D-lysine, heated in oven at 56ºC for 1 h, deparaffinized using 3 baths of xylol and rehydrated with 2 baths of ethanol 100% and 1 bath of ethanol 95% (5 min in each step). Next, they were submitted to 2 baths of phosphate buffered saline (PBS, pH=7.2-7.4) for 5 min. Endogenous peroxidases and serum proteins were inactivated for 15 min with 3% hydrogen peroxide and serum-free protein block, respectively, and washed in PBS. The recovery of the epitopes of protein antigens was accomplished by heating the sections at 95ºC in a 10 mM sodium citrate buffer for 30 min and a new wash in PBS. Primary antibodies against human MMP-2 (AF902; R&D Systems, Minneapolis, MN, USA), MMP-9 (AF911; R&D Systems) and CD31 (SC-1506; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were diluted 1:250 in PBS containing 1% bovine serum albumin (BSA) and dropped upon the slides which remained in wet chamber at room temperature for 1 h. Then the sections were washed in PBS, aspirated and exposed to the biotinylated secondary antibody rabbit anti-goat IgG-HRP (E066; Dako Cytomation Inc., Mississauga, ON, Canada) at room temperature for 15 min. All sections were washed three times in PBS and incubated in streptavidin-peroxidase for 30 min. The negative control was obtained by substituting the primary antibody with PBS/BSA in each batch. The slides were washed tree times with PBS, and after aspiration of the excess, they were exposed to a chromogenic substrate 3,3'-diaminobenzidine (DAB; Dako Cytomation Inc.) for 5 min. The slides were washed with distilled water, counter-stained with Harris Hematoxylin for 1 min and the excess removed with rinse in distilled water. Additionally, 3 shorter baths of ethanol 100%, 3 baths of xylol and bonding of coverslips with syntethic resin Entellan (Merck, Darmstadt, Germany) completed the procedure.

Histological and Histomorphometric Analyses of Immunostainings

The immunohistochemical preparations were evaluated by a person unaware of the clinicopathological status of the patients. Photomicrographs of non superimposed contiguous fields were obtained with a digital camera (Cybershot DSC-W300; Sony, Manaus, AM, Brazil) connected to an optical microscope (FWL-1000; Feldman Wild Leitz, Manaus, AM, Brazil) and analyzed using Image Pro-Plus 6.0 (MediaCybernetics, San Diego, CA, USA), calibrated in micrometer/pixel and with a virtual 100-point grade for counting of immunostained dots.

MMP-2 and MMP-9 expressions were evaluated in photomicrographs of SCC with ×200 magnification in 20 fields *per* slide. The number of points that coincided with positive marking for MMPs was counted in each area. The representative average percentage of the immunostained fields in each lesion was obtained. Values above 10% were considered positive in the classification of MMP status.

The CD31/MVD expression was determined in photomicrographs of SCC with total ×400 magnification in 5 fields from area of highest vascular density in the tumor *per* slide. Vessels up to 50 µm in diameter were considered for counting. Isolated cells or groups of vascular cells without a visible lumen were not included.

Statistical Design

The numeric variables and the frequency distribution of the category variables were evaluated using descriptive statistics of measurements with central tendencies and variability. The non-parametric Mann-Whitney U test was used to evaluate the association between the numeric variables and the markers, and the chi-square test of frequencies was applied to the contingency tables. Fisher's exact test was applied when at least one value was less than 5 in 2x2 tables. The probabilities of overall survival were estimated by the Kaplan-Meier method and the comparison of survival curves was carried out using the logrank test. The follow-up period was defined as the interval between the date of surgery and the date of either death or the last status information. A significance level of 5% was applied to all statistical tests.

Results

In the obtained sample, there was a predominance of men and poor survival rate. Thirty-six subjects were males and 5 were females and their ages ranged from 39 to 95 years (mean age: 54.8 years). At the last check of the records, only 4 subjects were alive, 24 had died of cancer, 8 had died from other causes, and 5 subjects were lost to follow-up. The shortest follow-up was 12 days and the longest was 8,171 days, with a mean follow-up period of 1,897 days. More than one half (51.2%) of the tumors were well differentiated, 36.6% were moderately differentiated and 12.2% were poorly differentiated. Approximately 80% of the subjects were in an advanced clinical stage (III and IV) and 56.1% had lymph node metastases. The number of vessels found *per* slide ranged from 20 to 46, with an average of 21.3 ± 7.8 used as an analysis parameter of MVD.

All three biomarkers were positive for SCC of floor of the mouth. MMP-2 had stronger staining for fibroblasts in the tumor stroma and weaker for epithelial tumor cells and inflammatory cells. MMP-9 expression was concentrated in the macrophage-rich inflammatory infiltrate but had also weak staining for fibroblast-like cells and tumor cells in the basal layer of epithelium. CD31 staining vascular endothelium aided identification of blood microvessels and obtaining the value of MVD (Fig. 1).

The distribution of demographic and clinical variables according to MMP-2 (Table 1) and MMP-9 expressions (Table 2) showed labeling with MMP-2 correlated to N positive stage, indicating lymph node metastasis at the time of initial diagnosis while the positivity for MMP-9 is not related to the demographic data (p>0.05). The analysis of demographic and clinical variables in function of MVD showed a lower degree of embolization (Table 3). The presence of only one case of bone invasion this category inappropriate for the assessment of correlation with molecular variables and the prognosis of patients.

Table 1. Distribution of demographic variables, TNM staging and histopathological variables for MMP-2 immunostaining

Variables and categories	Negative Frequency (%)	Positive Frequency (%)	p value
Gender			
Male	17 (42.2)	19 (52.8)	0.999*
Female	2 (40)	3 (60)	
Age of diagnosis			
N	19	22	
Minimum-maximum	42-95	39-69	0.084**
Median	56	53.5	
Average ± SD	57.8 ± 11.9	52.2 ± 8.3	
T stage			
T1+T2	9 (45)	11 (55)	0.867
T3+T4	10 (47.6)	11 (52.4)	
N stage			
N0	12 (66.7)	6 (33.3)	0.030*
N positive	7 (30.4)	16 (69.6)	
Tumor grade			
Poorly + Moderately (II+III)	10 (50)	10 (50)	0.678
Well differentiated (I)	9 (42.9)	12 (57.1)	
Perineural invasion			
Negative	12 (42.9)	16 (57.1)	0.511
Positive	7 (53.8)	6 (46.2)	
Embolization			
Negative	13 (43.3)	17 (56.7)	0.524
Positive	6 (54.5)	5 (45.5)	
Bone invasion			
Negative	18 (45)	22 (55)	NA
Positive	1 (100)	0 (0)	

SD: standard deviation; NA: not applicable; p: Chi square frequency test; *p: Fisher's exact test; **p: Mann-Whitney U test.

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Analysis of distribution in negative and positive cases among molecular variables showed no correlation of MMPs expression with MVD. However, there was negative correlation between MMP-9 and MMP-2 (p=0.018, Mann-Whitney U test, data not shown).

The relationship between the analyzed variables and patient survival is shown in Figure 2. The 5-year survival rate was 36.6% and the 10-year rate was 30.6%. The logrank test revealed that positive N stage, a poorly differentiated histological grade, the perineural invasion and MMP-9 expression were associated with poorer survival rates.

Discussion

Tumorigenesis and tumor progression are based on at least seven fundamental changes in cell physiology: 1) production of signs of autocrine growth; 2) insensitivity to signs of growth inhibition; 3) escape from apoptosis; 4) loss of senescence; 5) maintenance of angiogenesis; 6) tissue invasion; 7) metastases (7). The ability to invade tissues and establish colonies in remote sites certainly are characteristics that define malignant neoplasms. Since metastases are the main cause of death in cancer patients, a better understanding of the processes of tumor invasion and metastasis is indispensable and it can lead to the identification of new forms of treatment (3).

There is no consensus about the cell types most directly involved in the production of MMP-2 and MMP-9 in the tumor progression. Some authors state that tumor cells produce higher levels of proteolytic enzymes than normal cells (8) or indirectly stimulate the adjacent stromal cells

Table 2. Distribution of demographic variables, TNM staging and histopathological variables for MMP-9 immunostaining

Variables and categories	Negative Frequency (%)	Positive Frequency (%)	p value
Gender			
Male	25 (69.4)	11 (30.4)	0.317*
Female	2 (40)	3 (60)	
Age of diagnosis			
N	27	14	
Minimum-maximum	40-95	39-67	0.783**
Median	54	55	
Average ± SD	55.48 ± 11.5	53.5 ± 8.1	
T stage			
T1+T2	15 (75)	5 (25)	0.228
T3+T4	12 (57.1)	9 (42.9)	
N stage			
N0	14 (77.8)	4 (22.2)	0.154
N positive	13 (56.5)	10 (43.5)	
Tumor grade			
Poorly + Moderately (II+II)	12 (60)	8 (40)	0.440
Well differentiated (I)	15 (71.4)	6 (28.6)	
Perineural invasion			
Negative	20 (71.4)	8 (28.6)	0.307*
Positive	7 (53.8)	6 (46.2)	
Embolization			
Negative	19 (63.3)	11 (36.7)	0.709*
Positive	8 (72.7)	3 (27.7)	
Bone invasion			
Negative	27 (67.5)	13 (32.5)	NA
Positive	0 (0)	1 (100)	

SD: standard deviation; NA: not applicable; p: Chi square frequency test; *p: Fisher's exact test; **p: Mann-Whitney U test.

Table 3. Distribution of demographic variables, TNM staging and histopathological variables for CD31/MVD immunostaining

Variables and categories	≤ 21 Frequency (%)	> 21 Frequency (%)	p-value
Gender			
Male	18 (50)	18 (50)	0.999*
Female	2 (40)	3 (60)	
Age of diagnosis			
N	20	21	
Minimum-maximum	42-67	39-95	0.140**
Median	57	53	
Average ± SD	56 ± 6.9	56.7 ± 12.9	
T stage			
T1+T2	12 (60)	8 (40)	0.161
T3+T4	8 (38.1)	13 (61.9)	
N stage			
N0	7 (38.9)	11 (61.9)	0.262
N positive	13 (56.5)	10 (43.5)	
Tumor grade			
Poorly + Moderately (II+II)	12 (60)	8 (40)	0.161
Well differentiated (I)	8 (38.1)	13 (61.9)	
Perineural invasion			
Negative	14 (50)	14 (50)	0.819
Positive	6 (46.1)	7 (53.9)	
Embolization			
Negative	10 (33.3)	20 (66.7)	0.001*
Positive	10 (91.9)	1 (9.1)	
Bone invasion			
Negative	19 (47.5)	21 (52.5)	DNA
Positive	1 (100)	0 (0)	

SD: standard deviation; NA: not applicable; p: Chi square frequency test; *p: Fisher's exact test; **p: Mann-Whitney U test.

to secrete them, opening a tissue space for tumor invasion (3,9). The production of MMP by the stroma can also be interpreted as a feature of epithelial tumors that have lost their ability to produce MMP due to the dedifferentiation process (3). The most accepted hypothesis is the main production of MMP-2 and MMP-9 by stromal tumor cells including fibroblasts, inflammatory cells and endothelial cells, playing an active role in carcinogenesis and tumor invasion (9) similar to the outcomes of this study, with marked expression of MMP-2 and MMP-9 in the stroma. It is important to highlight the participation of the
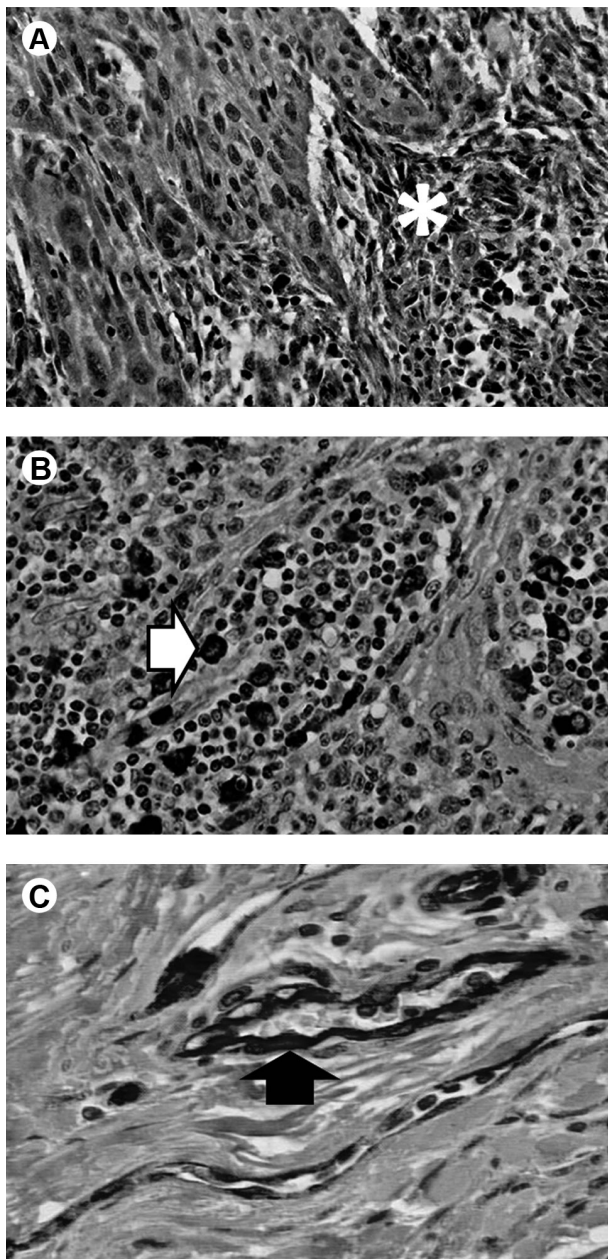

Figure 1. Immunohistochemical evaluation of SCC of the floor of the mouth. A: Positive staining for MMP-2 in the connective stroma (*). B: MMP-9 expression in an intense, macrophage-rich inflammatory infiltrate in the stroma (white arrow). C: CD31 expression in endothelium of a blood microvessel (black arrow). Amplification: 20× (A, B); 40× (C).

inflammatory component of a neoplasm, which includes a diverse cell population (neutrophils, dendritic cells, macrophages, eosinophils, mast cells and lymphocytes) able to producing cytokines, mediators, proteases and MMPs, keeping close correlation with modulation of extracellular matrix degradation and tumor invasion (2,10).

MMP-2 was more strongly immunostained in fibroblast-like cells of tumor stroma. Fibroblasts are important in cancer progression due to release of MMP-2 on a larger scale and facilitation for the tumor invasion and metastasis (11). MMP-2 in this work was a rare condition in inflammatory cells. This suggests that the invasion afforded by MMP-2 is not influenced by local inflammation close to the tumor. The significant relationship between MMP-2 and lymph node metastasis corroborates other findings (3). A less expressive staining for MMP-2 in the basal membrane could suggest that tumor cells stimulate the MMPs expression in their environment to promote catabolism of extracellular matrix (type IV collagen) by the phagocytic host cells, paving the way for the tumor expansion (11).

Predominant immunostaining for MMP-9 in inflammatory cells does indicate that an expressive inflammatory reaction around the tumor increases the MMP-9 concentration and the risk of tumor invasion resulting from the action of gelatinase. A greater dissolution of extracellular matrix creates space for the tumor mass, facilitates intratumoral angiogenesis, decreases cellular adhesion and sustains the metastasis (12). However, one might consider that chronic inflammation as the body's normal reaction can promote the release of compounds that prevent tumor progression (10). MMPs also influence the angiogenesis promoting release of pro-angiogenic molecules (VEGF and angiopoetin) and anti-angiogenic molecules (angiostatin) (11). In particular, MMP-9 action also generates tumstatin and endostatin, cleavage fragments of the alpha-3 chain of type IV collagen and alpha-1 chain of type XVIII collagen with anti-angiogenic properties (12). For this dual influence in the mechanism of angiogenesis, it was considered plausible the lack of correlation between MMPs and MVD studied in this work, corroborating other similar results (13). On the other hand, the relationship found between MMP-9 and poor prognosis refutes other findings (14) and deserves further study.

Tumor vascularization is an essential factor for tumor progression. Without angiogenesis, the tumor mass cannot exceed a critical size and is restricted to a tissue diffusion distance of about 0.2 mm (15). In the present study, no relationship was found between MVD and clinical or histological parameters such as T or N stage, histological grade, perineural invasion or patient survival. Other authors have equally failed to find such associations in SCC of head and neck, which may indicate that the tumor

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physiopathology is more complex than the mere presence or absence of vessels, and the interaction of various factors that influence tumor progression makes it difficult to objectively evaluate the effect of each individual factor (4,16). This behavior could be explained in the use of hot spot method, highly variable for counting of MVD in low-vascularization regions between SCC islets leading to misinterpretation (4), or due to unspecific immunostaining of newly formed blood vessels and pre-existing ones, disabling its prognostic importance (16). Furthermore, the fact that different sites have different degrees of vascularization may hinder the evaluation of recurrence and metastasis rates (4,6,17). The present study comprises only SCCs of the floor of the mouth and yet no such correlations were found, as opposite to other studies with correlation between MVD and tumor aggressiveness (13), tumor size (18) or tumor grade (19). There was a negative correlation between MVD and embolization, which may be attributed to a deficiency of the newly formed vessels in performing their usual functions, such as gas exchange and supplying nutrients to the neoplasm (15). Furthermore, the presence of endothelial cells may favor tumor invasion through cytokine production, thus creating a chemically attractive environment for tumor cells (20). On the other hand, if one considers that new vessels formed in an uncontrolled way in a tumor mass are not always functional, their capabilities of chemoattraction and of supplying nutrients to the tumor may be impaired, thus hindering the penetration of tumor cells and explaining the lack of correlation between the MVD and the other evaluated parameters.

The literature attempts to establish an association between some histological variables and SCC prognosis. Factors such as lymph node metastases and histological grade have a clear correlation with cancer prognosis (2,21,22). The present study found a significant correlation between survival, lymph node metastases, more aggressive histological grades (II and III) and perineural invasion. The latter has been reported as a negative prognostic factor in the development of SCC (2,5). Tumor propagation could occur through the perineural connective tissue that envelops the peripheral nerves because of its low resistance (23). Other authors reported that malignant tumors of the floor of mouth with perineural space invasion are more prone to metastasize to cervical lymph nodes, suggesting higher aggressiveness and more recurrences (24). The overexpression of neural growth factor in salivary gland carcinomas may be a reason for their affinity for nerves, perhaps being a marker for perineural invasion in such lesions in humans (25). Although the present study has shown a significant correlation between perineural invasion and poor survival, data that could aid in the selection of a more effective therapy, more studies are needed in order to determine the molecular mechanisms involved in this process.
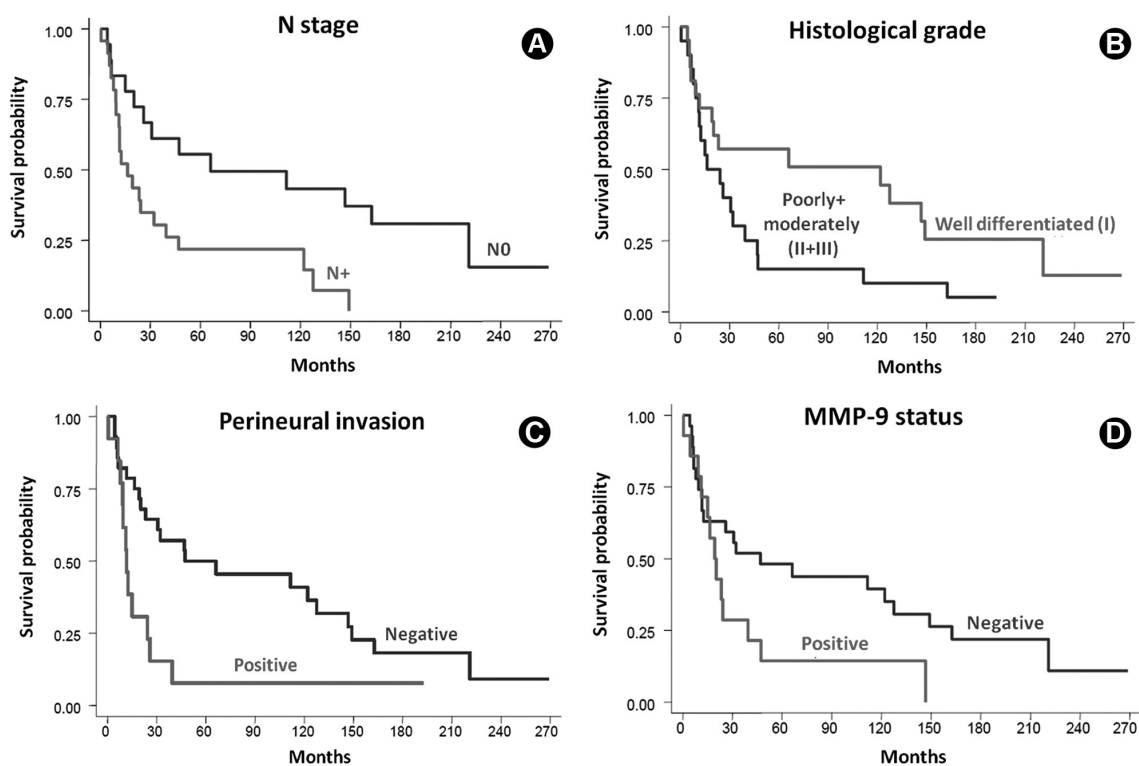

Figure 2. Kaplan-Meier survival estimates according to: (A) N stage, (B) histological grade, (C) perineural invasion and (D) MMP-9 status.

Based on the results obtained in SCC of the floor of the mouth, it is possible to conclude that MMP-2 expression could be indicative of lymph node metastasis and MMP-9 is associated with lower probability of survival. There was no significant association between immunoexpression of CD31/MVD and the other variables.

Resumo

O objetivo deste estudo foi avaliar a imunoexpressão de MMP-2, MMP-9 e CD31/densidade microvascular em carcinomas espinocelulares de soalho bucal e correlacionar os resultados com variáveis demográficas, de sobrevida, clínicas (estadiamento TNM) e histopatológicas (grau de diferenciação tumoral, invasão perineural, embolização e invasão óssea). Dados de prontuários e de diagnósticos de 41 pacientes foram revisados. Cortes histológicos foram submetidos à imunomarcação usando anticorpos primários para MMP-2, MMP-9 e CD31 humanos e sistema streptoavidina-biotina-imunoperoxidase. Análise histomorfométrica quantificou a positividade para MMPs (20 campos, grade de 100 pontos por lâmina, ×200) e para CD31 (microvasos <50 μm na área de maior vascularização, 5 campos, grade de 100 pontos por lâmina, ×400). O planejamento estatístico foi composto pelo teste não paramétrico U de Mann-Whitney (verificação da associação entre variáveis numéricas e imunomarcações), teste de frequências do qui-quadrado (em tabelas de contingência), teste exato de Fisher (quando pelo menos uma frequência esperada foi menor do que 5 em tabelas 2x2), método de Kaplan-Meier (estimativa de probabilidades de sobrevida global) e teste de logrank (comparação das curvas de sobrevida), todos com nível de significância de 5%. Houve correlação estatisticamente significante entre imunomarcação para MMP-2 e metástase em linfonodo. Os fatores relacionados negativamente com a sobrevida foram estadiamento N, gradação histopatológica, invasão perineural e imunomarcação de MMP-9. Não houve associação significativa entre imunoexpressão de CD31 e as demais variáveis. A intensidade de imunomarcação para MMP-2 pode ser indicativa de metástase em linfonodo e para MMP-9 de uma menor probabilidade de sobrevida.

Acknowledgements

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