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Collagen IV and laminin expression in squamous cell carcinomas of lower lip and tongue

Expressão de colágeno IV e laminina em carcinomas de células escamosas de lábio inferior e língua

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ABSTRACT

Objective: In this study, the expression of extracellular matrix proteins was immunohistochemically studied and compared with the histological grading of squamous cell carcinomas of the lower lip and tongue. **Material and Methods:** The lower lip carcinomas (n = 12) and the tongue carcinomas (n = 12) were histopathologically graded according to Bryne's method. The immunohistochemical technique used specific antibodies for collagen IV and laminin. Histopathological and immunohistochemical analysis were carried-out at the invasive tumor front. **Results:** Most of the lower lip carcinomas (91.7%) were classified with lower scores and all tongue carcinomas (100%) with high-grade malignant scores ($p < 0.01$). Collagen type IV expression was absent in the peritumoral basement membrane in 50% of lower lip carcinomas and in 66.7% of tongue carcinomas ($p = 0.09$). Laminin expression was absent in the peritumoral basement membrane in 66.7% of lower lip carcinomas and in 58.3% of tongue carcinomas ($p = 0.48$). When these two glycoproteins were expressed, they showed a linear, thin and discontinuous pattern and a weak intensity of expression. **Conclusion:** The high-grade malignancy score of the tongue carcinomas was associated with the pattern of expression of the matrix proteins studied. This suggested that tongue squamous cell carcinomas have more invasive potential and more aggressive biological behavior than the lower lip carcinomas.

KEYWORDS

Collagen type IV; Carcinoma; Immunohistochemistry; Laminin.

RESUMO

Objetivo: Neste estudo, a expressão das proteínas da matriz extracelular foi estudada imunoistoquimicamente e comparada com a classificação histológica dos carcinomas de células escamosas do lábio inferior e língua. **Material e Métodos:** Os carcinomas de lábio inferior (n = 12) e os carcinomas de língua (n = 12) foram graduados histopatologicamente de acordo com o método de Bryne. A técnica de imunoistoquímica utilizou anticorpos específicos para colágeno IV e laminina. Análises histopatológica e imunoistoquímica foram conduzidas na frente invasiva tumoral. **Resultados:** A maioria dos carcinomas de lábio inferior (91,7%) foi classificada em baixo grau e todos os carcinomas de língua (100%) em alto grau de malignidade ($p < 0,01$). A expressão de colágeno tipo IV estava ausente na membrana basal peritumoral em 50% dos carcinomas de lábio inferior e em 66,7% dos carcinomas de língua ($p = 0,09$). A expressão de laminina estava ausente na membrana basal peritumoral em 66,7% dos carcinomas do lábio inferior e em 58,3% dos carcinomas de língua ($p = 0,48$). Quando estas duas glicoproteínas foram expressas, mostraram-se com um padrão linear, fino e descontínuo e uma fraca intensidade de expressão. **Conclusão:** O alto grau de malignidade dos carcinomas de língua associou-se com o padrão de expressão das proteínas de matriz estudadas. Isso sugere que carcinomas de células escamosas de língua têm comportamento biológico mais agressivo e potencial mais invasivo do que os carcinomas de lábio inferior.

PALAVRAS-CHAVE

Carcinoma; Colágeno tipo IV; Imunoistoquímica; Laminina.

INTRODUCTION

The majority of oral cancers are squamous cell carcinomas (SCC), especially affecting the tongue and lower lip [1]. Clinical, morphological and immunohistochemical studies have demonstrated that the squamous cell carcinomas with poor prognosis show higher proliferative activities, lower levels of differentiation, more vascularization and higher potential for invading and spreading to the adjacent and distant tissues [2-5].

The first stage in the metastasis of oral SCC involves destruction of the basement membrane (BM) and invasion into the submucosal tissue [6]. The main components of the BM are laminin, collagen type IV, and in a smaller proportion, other molecules. In general the BM is lost in many invasive carcinomas [7].

The dissemination of SCC elsewhere is possible, and they lose their intercellular adhesion and initiate the migration mechanism into the basal lamina [8]. This migratory mechanism depends on the rupture of the BM, whose role in carcinoma biology has not yet been clarified [8]. In a similar manner, the carcinoma cells migrate into the tumor stroma degrading the interstitial collagen [9,10-12]. At this time, these carcinoma cells express superficial superficial laminin receptors, with the aim of penetrating more easily into the adjacent connective tissue [13-15].

Immunohistochemical analysis of BM components can be useful for evaluating tumor invasion and metastasis in SCC [16]. Based on this information, the objectives of this study were to verify the extracellular matrix protein expression (collagens IV, laminin) in SCCs with different histological gradings and anatomical sites (lower lip and tongue).

MATERIAL AND METHODS

The study was approved by the Ethics Committee of the Federal University of Jequitinhonha and Mucuri Valleys under Protocol Number 122/2010, and was conducted in accordance with the Declaration of Helsinki 1975, revised in 2008.

Sample size was calculated based on the prevalence (1.4%) of squamous cell carcinomas [17] with the margin of error set at 5%. The minimum sample size required was 21 SCCs considering a 95% confidence level and 80% power.

In this experiment, tissue specimens of 12 SCC of the lower lip and 12 of the tongue were obtained from the biopsy files of the Oral Pathology Archives, in the Dentistry Department of the Federal University of Jequitinhonha and Mucuri Valley and Federal University of Rio Grande do Norte, Brazil. Patients were surgically treated without prior radiotherapy or chemotherapy. Samples were excluded from the study in case of incisional biopsy, specimens with inadequate material or extensive areas of necrosis.

Histopathology Sections (5 mm) were stained with hematoxylin–eosin (HE) for evaluation of the tumor invasion front. After this, the carcinomas were classified according to the histological grading system proposed by Bryne [3] that considers four parameters for the determination of the grade of malignancy: degree of keratinization, nuclear pleomorphism, invasion pattern, and inflammatory infiltrate. This system was adapted by the author from Bryne's first classification system that included five morphological parameters. In view of the reduction in the number of parameters evaluated by the Bryne method [3], for this study, the classification of cases was adapted to being based on the sum of scores, with cases presenting 4–8 points being classified as low-grade and those with more than 8 points being classified as high-grade of malignancy.

For the immunohistochemical study, the tissue sections were deparaffinized and immersed in 3% hydrogen peroxide to block endogenous peroxidase activity. The tissue sections were

then washed in phosphate-buffered-saline (PBS). Tissue antigenic recovery was leaded with 1% pepsin for collagen IV and laminin at 37oC for 60 min. Endogenous peroxidase was blocked by 0.1% (v/v) H2O2 / methanol at room temperature for 10 min. Sections were washed for 10 min in running tap water and 10 min in tris/HCl buffer (trihidroxymetylaminomethane) (Laborsynth, São Paulo, Brazil) pH 7.4. The primary antibodies for collagen IV (CIV22 clone) (Dako, Glostrup, Denmark) and laminin (LAM-89 clone) (Sigma Chemical, St. Louis, USA) were diluted in tris/HCl buffer (pH 7.4) and 1% bovine serum albumin (BSA). The sections were incubated in a humidified chamber with 1:20 collagen IV antibody and 1:800 laminin antibody at room temperature for 120 min. After washing 2 times for 5 min each in tris/HCl buffer, the second antibody used was a biotinylized rabbit IgG to mouse IgG at a dilution 1:100 for 30 min, at room temperature. The tissue sections were then washed twice in PBS and treated with streptoavidin-biotin-peroxidase complex (Dako, Glostrup, Denmark) at room temperature in order to bind the primary antibodies. Peroxidase activity was visualized by immersing tissue sections in diaminobenzidine (D5637) (Sigma Chemical, St. Louis, USA), resulting in a brown reaction product. Finally, tissue sections were counterstained with Mayer's hematoxylin and coverslipped. Positive controls for laminin and collagen IV were sections of normal oral mucosa. As negative controls, samples were treated as above, except that the primary antibody was replaced by a solution of bovine serum albumin (BSA) in PBS. Under a light microscope the staining patterns of the studied proteins were evaluated considering location, tickles and continuity of immunostaining in extracellular matrix. The intensity of protein expression was graded as weak, moderate or intense.

The data relating to observation of SCC were compiled for the SPSS® software (IBM Inc., Chicago, USA) version 22.0 in order to perform statistical analysis. A descriptive statistical analysis was performed. The association between qualitative variables was verified by Chi-square test (X^2). Also, p-value less than 0.05 was considered statistically significant.

RESULTS

The histological grading demonstrated that the majority of the lower lip carcinomas (91.7%) have low-grade malignancy and all cases of tongue carcinomas (100%) have high-grade malignancy (Figure 1, Table 1).

Table 1 - Malignancy grading of the squamous cell carcinomas of the lower lip and tongue

	High-grade malignancy		Lower-grade malignancy		p*
	n	%	n	%	
Lower lip	01	8.33%	11	91.67%	<0.001
Tongue	12	100%	-	-	

* Chi-square Test

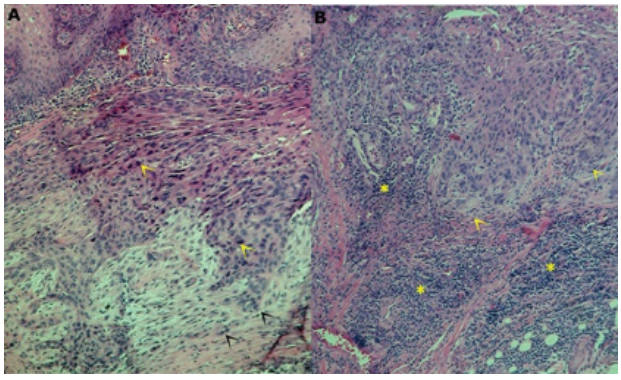

Figure 1 - Invasive front of squamous cell carcinoma. (A) High-grade malignant tumor shows diffuse invasive pattern with little nests and individual cells (black arrows), nuclear hyperchromatism and pleomorphism (yellow arrows), absence of inflammatory infiltrate. (B) Low-grade malignant tumor shows well limited invasive pattern (yellow arrows) and intense inflammatory infiltrate (yellow asterisks). (HE, original magnification x100).

Statistically significant differences were observed in degree of keratinization ($p = 0.03$), nuclear polymorphism ($p = 0.02$) and leucocyte infiltration ($p = 0.02$) among squamous cell carcinoma of lower lip and tongue. There were no significant difference in pattern of invasion (p

= 0.10). Moreover, there were highly significant differences between the malignancy grading of these types of carcinomas, when the total score of malignancy was compared ($p < 0.01$).

In the immunohistochemical analysis, collagen IV showed absence of immunoreactivity in the basement membrane of the cellular neoplastic sheets in 50% of the lower lip and in 66.7% of the tongue carcinomas (Table 2). The laminin expression was absence in 67.3% of the lip and in 58.3% of the tongue carcinomas (Table 2). When these two glycoproteins were present in the peritumoral basement membrane, they showed weak intensity of expression in a linear, thin and discontinuous pattern (Figure 2).

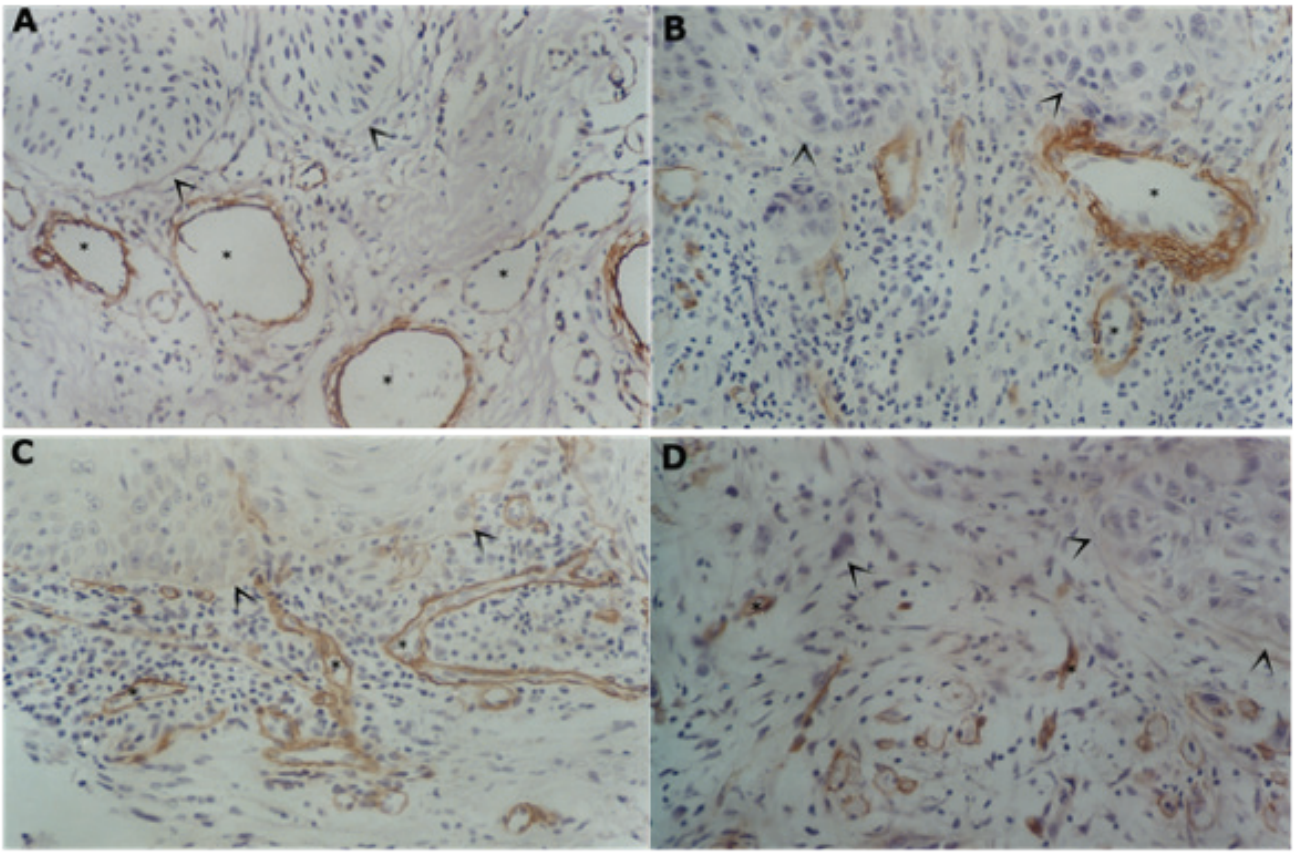

Figure 2 - Collagen and laminin present in peritumoral basement membrane. Scanty immunorreactivity of the (A) collagen IV and (C) laminin at the invasive front of the lower lip squamous cell carcinoma. Absence of immunorreactivity of the (B) collagen IV and (D) laminin at the invasive front of the tongue squamous cell carcinoma. (SABC-immunostain, original magnification x200). Arrows indicate peritumoral basement membrane; asterisks indicate blood vessel.

Table 2 - Immunohistochemical expression of collagen IV and laminin in the peritumoral basement membrane of lower lip and tongue squamous cell carcinomas

	LOWER LIP CARCINOMAS					TONGUE CARCINOMAS				
	Immunoreactivity		Intensity			Immunoreactivity		Intensity		
	Positive	Negative	Weak	Moderate	Intense	Positive	Negative	Weak	Moderate	Intense
Collagen IV	6 (50%)	6 (50%)	3 (50%)	3 (50%)	–	4 (33.3%)	8 (66.7%)	4 (100%)	–	–
Laminin	4 (33.3%)	8 (66.7%)	1 (25%)	2 (50%)	1 (25%)	5 (41.7%)	7 (58.3%)	2 (40%)	3 (60%)	–

The intensity of collagen IV expression between SCC of lower lip and tongue showed a trend towards statistical significance ($p = 0.09$), but this tendency was not observed for laminin expression ($p = 0.48$).

DISCUSSION

Many studies have been developed about the biological behavior of the oral SCC, seeking to establish histomorphological parameters for systems to grade the malignancy of this carcinoma and aiming to find new prognostic indicators. As regards the degree of keratinization, nuclear polymorphism, leucocyte infiltration and pattern of invasion, the present study observed that the all tongue squamous cell carcinomas were high-grade malignant tumors compared with the lower lip carcinomas that were predominantly of low-grade malignancy.

These results are in agreement with the studies of Broders [18], Lund et al. [19], Lund et al. [20], Bryne [21] and Visscher et al. [22]. The histomorphological and statistical results of malignancy grading ($p < 0.01$) of this study suggested that the tongue SCC are biologically more aggressive than the lower lip squamous cell carcinomas. These observations have also been demonstrated by several other studies [1,19,20,22-24].

The present study showed statistically significant difference in degree of keratinization, nuclear polymorphism and leucocyte infiltration when lower lip and tongue SCC were compared. This data can be a reflection of findings that have been reported in the literature: that the aggressiveness of these tumors depends on a series of factors, and tongue carcinomas generally tend to have a more aggressive biological behavior [1,25-27].

The present immunohistochemical results demonstrated absence of immunostaining for the collagen IV in the peritumoral BM in 50% of the lower lip carcinomas and in the majority of the tongue carcinoma cases studied (66.7%) ($p = 0.09$). These results corroborate the findings demonstrated by Wilson et al. [28], Hagedorn et al. [29] and Zargaran et al. [16]. These immunohistochemical aspects can have an effect on the action of proteolytic enzyme engineering by the neoplastic cells that induce lysis and degradation of collagen IV, during the process of tumor invasion.

The absence of laminin immunoexpression was observed in the peritumoral BM of the majority of lower lip (66.7%) and tongue carcinomas (58.3%) ($p = 0.48$). These findings are similar to those in the studies of Firth and Reade [30], Haas et al. [31] and Mostafa et al. [9] and according to these authors, the more undifferentiated carcinomas show a lower level of laminin staining.

Findings contrary to the results of the present study were demonstrated by Hagedorn et al. [29] who observed an increased deposition of laminin in the peritumoral BM and intracytoplasmic staining of laminin in the neoplastic cells of oral SCC. Controversial results on laminin expression in SCC can be a reflection of different interactions between the neoplasic cells and the tumor stroma, depending on the stage of tumor development.

According Santos-García et al. [7], the ability of malignant neoplasias to destroy the BM has been correlated with their invasive potential and the loss of continuity of laminin and collagen IV. In the present study, a joint evaluation was made of the pattern of immunohistochemical expression of the proteins studied in the basement membrane of the malignant neoplastic epithelial nests at the tumor invasion front. This suggested that collagen IV and laminin are degraded during the tumor invasion process, making it easy for the malignant neoplastic cells to migrate.

CONCLUSION

Within the limits of this study, the results help towards better comprehension of the

molecular mechanisms involved in migration and invasion by the neoplastic cells of the oral SCC. This contributes to the establishment of new prognostic indicators of this carcinoma. In conclusion, the differential immunostaining pattern of the studied glycoproteins in the lower lip and tongue SCC associated with the high-grade malignancy score demonstrated by the tongue SCC, suggested that the tongue carcinomas have higher invasive potential and a more aggressive biological behavior than the lower lip carcinomas.

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