Immunohistochemical Study of Laminin-332 γ2 Chain and MMP-9 in High Risk of Malignant Transformation Oral Lesions and OSCC

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ABSTRACT

Objectives: Oral squamous cell carcinoma is associated with alterations in basement membrane. Laminin-332 is present in basal lamina and performs multiple biologic effects by γ2 chain. Matrix metalloproteinase acts disrupting extracellular components and was related to poor prognosis in cancer. Here, molecular profile of laminin-332 γ2 chain and matrix metalloproteinase-9 was assessed in oral lesions.
Material and Methods: The expression of laminin-332 γ2 chain and matrix metalloproteinase-9 (MMP-9) was examined by immunohistochemistry in 10 patients with high risk of malignant transformation oral lesions and 26 cases of oral squamous cell carcinoma (OSCC). Associations between microscopic and clinicopathologic features were established.
Results: Immunostaining of laminin-332 γ2 chain in high risk oral lesions was most detected in basement membrane which is continuous, while the majority of OSCC cases showed a discontinuous membrane ($P = 0.001$). It was observed a positive reaction for γ2 chain in invasive fronts and a higher expression in epithelial compartment of smoking patients with OSCC ($P < 0.0001$). In epithelium, MMP-9 expression was presented in all layers with no difference between lesions. However, an elevated immunostaining in stromal cells was associated with male patients ($P = 0.0054$), older than 60 years ($P = 0.0101$) and with OSCC.
Conclusions: Present study results support the hypothesis of changes in molecules expression in high risk oral lesions and oral squamous cell carcinoma. A relation between clinical and molecule profile was observed. Those molecules may represent a useful tool to predict oral cancer behaviour.

Keywords: carcinogenesis; laminin; matrix metalloproteinase 9; oral cancer; oral pathology; squamous cell carcinoma.

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INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the most common oral cancer with 90% of the cases [1]. Tobacco is the main risk factor, and OSCC is linked to excessive consumption of alcohol and HPV infection [2]. OSCC may arise from oral potentially malignant disorders (OPMDs). Clinically, oral leukoplakia is one of the most common type of OPMD, with a malignant transformation rate between 2 - 3% per year [3,4]. Histopathologically, oral epithelial dysplasia (OED) is the most important feature of OPMDs and was considered as the progenitor for malignant changes [5]. Regarding cancer progression, one important step is the alteration that affects basement membrane, a dynamic structure composed of type IV collagen, proteoglycans and a dense network of glycoproteins [6]. Laminin-332 is a large extracellular glycoprotein presents in basal lamina of epithelial cells. This molecule performs multiple biologic effects, such as cell attachment, migration, differentiation and proliferation [7]. Recent studies have shown that biological activity of laminin-332 is modulated by γ2 chain process [8,9]. Matrix metalloproteinase (MMP) acts disrupting extracellular components which result in changes of cell-cell and cell-matrix interactions [10]. Among the various types of MMPs, MMP-9 has been highlighted in the context of tumorigenesis. Those enzymes were considered the new biomarkers for cancer in many sites, such as breast (MMP-1, -9, -13), lungs (MMP-1, -7, -9) and colorectal area (MMP-1, -2, -7, -9, -13) [11-13].

Clinic and morphologic parameters of OSCC are well defined by literature and histologic grading has been applied to define oral cancer behaviour for years, however, is important to emphasize that its prognostic value is still considered controversial. Thus, our objective was to assess the laminin-332 γ2 chain and matrix metalloproteinase-9 expression in high risk of malignant transformation oral lesions and oral squamous cell carcinoma. Also, the microscopic features were associated to patient's clinical data and its possible implication in carcinogenesis.

MATERIAL AND METHODS

Patients and tissue specimens

The retrospective study was approved by Ethics Committee of the Institution where it was developed (protocols 45995815.9.0000.5060 - Federal University of Espírito Santo/UFES, Brazil) and was performed in accordance with the Declaration of Helsinki. Paraffin embedded tissues from different sites were selected from the archives of the Oral Pathology Laboratory of Dentistry School/UFES between 2004 and 2011. A total of 36 lesions were selected and the confirmation of initial diagnosis were performed by oral pathologist (L.A.P.B.). The architectural and cytological scoring criteria for OED were applied in OPMD to define groups. The group of high risk oral lesions was consisted by 5 cases diagnosed microscopically with severe OED in accordance with the binary system proposed by Kujan et al. [5] for potential susceptibility for malignant transformation and 5 cases of CIS. The OSCC group consisted by 26 invasive OSCCs (9 well differentiated, 11 moderately differentiated and 6 undifferentiated). Cases of inflammatory fibrous hyperplasia (IFH) was included as a positive/negative control of the pattern expression of the studied molecules. Clinical data were collected from medical records and included: gender, age, lesion site and history of tobacco exposure. Exclusion criteria were: incomplete clinical data and insufficient sample for microscopic analysis.

Immunohistochemistry

For immunohistochemistry, 3 μm sections were submitted to the immunoperoxidase method. The primary antibodies used were: mouse anti-γ2 chain laminin-332 with dilution 1:100 (clone B-2: sc-25341, Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit anti-MMP-9 with dilution 1:500 (whole molecule-ab 38898, Abcam). Immunodetection was performed with the Reveal System (Spring/Biogen SPB-999) using 3,3′-diaminobenzidine as the chromogen. The sections were counterstained with Mayer's haematoxylin. Heat citrate buffer pH 6.0 was used for antigen retrieval and 1% bovine serum albumin (BSA) was applied for blocking non-specific antigens. The sections were incubated with the respective primary antibodies overnight in a humidified chamber maintained at 4 °C. Then, slides were depleted of endogenous peroxidase by incubating for 20 min with 3% hydrogen peroxide in room temperature. Subsequently, specimens were incubated with secondary antibody detection system according manufacturer's instructions. Positive (IFH) and negative controls (omission of primary antibody) were included.

Microscopic analysis

Immunostained sections were analysed and scored in a blind manner with respect to clinical information by a single investigator, previously calibrated (kappa 0.8)

using Olympus AX70 microscope (Olympus America Inc., NY, USA) with a digital camera Zeiss AxioCam ERC5s (Carl Zeiss Vision GmbH, Germany) coupled and Axio Vision 4.2 Release 4.8.2 images program (Carl Zeiss Vision GmbH, Germany). The expression of laminin-332 γ2 chain was evaluated according to tissue sites: (1) basement membrane and (2) epithelial compartment. For the last one, cells were separated into a basal layer (BL) and a suprabasal layer (SL). The BL consisted of one to two cell layers that were closest and perpendicularly organized on the epithelial-matrix interface. The rest of the epithelial cells overlying BL were designated as SL [7]. Expression was still analysed in invasive front and tumour nests. Regarding basement membrane staining, the following aspects were considered: absence; continuity; discontinuity. All slides were evaluated at x100 magnification. In relation to MMP-9 analysis, stroma and epithelium (BL+SL) were also considered. The number of stromal cells expressing MMP-9 was divided by total fields (cells/fields: C/F ratio). After that, 3 groups were established: fewer than 25% of the labelled cells, 25 - 50% labelled and more than 50% of stained cells [14].

Association of clinical and microscopic data

After microscopic analysis and data collection, associations between molecules expression and clinical parameters were established. Each of those clinical data (age, gender, site and tobacco exposure) were examined and correlated with molecules expression according to criteria described above.

Statistical analysis

Chi-squared test was applied to evaluate clinical data. Chi-square Test, Fischer`s Exact Test and Two-way ANOVA were used to analyse correlations between molecules expression and clinical data. The results were considered significant when $P < 0.05$. GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA) was used for statistical analysis.

RESULTS

Clinical data

Male was the most affected gender i.e. 70% in high risk oral lesions and 88.5% in OSCC cases. Patients older than 60 years were more prevalent in high risk oral lesions (80%). In OSCC most patients were under 60 years (57.7%). Lesions affected different sites and was predominantly in the alveolar ridge. Most of patients related smoking, especially in OSCC (77%). Table 1 summarized clinical data.

Expression of laminin-332 γ2 chain and MMP-9

Immunostaining of laminin-332 γ2 chain in basement membrane demonstrated differences. In high risk oral lesions, all cases showed staining in basement membrane and 60% were presented as a continuous profile (Figure 1C). In OSCC, 69.2% of cases had a discontinuous or no visible membrane (Figure 1E, G). That difference was statistically significant ($P = 0.001$). Related to positive expression in epithelial cells, a preferential staining in BL+SL (Figure 1E, F) was observed with no statistical difference between lesions (Table 2). Laminin-332 expression was detected in OSCC predominantly in single cells, periphery tumour nests or tumour-stromal interface of invasive fronts (Figure 1G). All cases of OSCC with invasive fronts showed positive expression for laminin-332 γ2 chain in epithelial cells and a cellular membrane staining was remarkable (Figure 1G, arrowheads). When MMP-9 was analysed in epithelium, it was possible to observe the expression in all layers (Figure 1D, F) with no significant difference between lesions (Table 2). However, the analysis of stromal cells showed a higher ($P = 0.0086$) MMP-9 expression (50% cells/field) in OSCC (Figure 1H, Table 2). The positive control of laminin-332 was observed as a discrete and continuous linear basement membrane in IFH (Figure 1A, arrow). Positive expression of MMP-9 was mainly observed in endothelial cell of small vessels (Figure 1B, * symbol). Neither antibodies showed staining brownish granules in BL and SL.

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Association between clinical data and expression of laminin-332 γ2 chain and MMP-9

Associations between data showed positive results. When only smokers were analysed, those with OSCC had a higher expression of laminin-332 γ2 chain in BL and BL+SL when compared to high risk oral cases ($P < 0.0001$) risk oral cases (Figure 2A). The same was not observed in basement membrane. The analysis showed no difference of laminin expression between lesions of smokers (Figure 2B). Regarding MMP-9 expression, when only male patients were analysed, those with OSCC showed more stromal cells with citoplasmatic staining than high risk oral cases ($P = 0.0054$) (Figure 2C). The same pattern was observed in patients older than 60 years, with a higher number of stromal cells with MMP-9 expression in OSCC cases than high risk oral lesions ($P = 0.0101$) (Figure 2D).

Table 1. Clinical data and histologic status of studied groups

	Case	Histologic status	Gender	Site	Age (years)	Habit
High risk oral lesion	1	Severe OED	M	Alveolar ridge	67	S
	2	Severe OED	F	Lip	61	NS
	3	Severe OED	F	Alveolar ridge	66	S
	4	Severe OED	M	Alveolar ridge	77	S
	5	Severe OED	F	Tongue	47	NS
	6	CIS	M	Alveolar ridge	61	S
	7	CIS	M	Alveolar ridge	30	NS
	8	CIS	M	Tongue	61	S
	9	CIS	M	Alveolar ridge	64	S
	10	CIS	M	Alveolar ridge	68	NS
Oral squamous cell carcinoma	11	Well differentiated	M	Mouth floor	79	S
	12	Well differentiated	M	Mouth floor	45	S
	13	Well differentiated	M	Alveolar ridge	52	S
	14	Well differentiated	M	Mouth floor	53	S
	15	Well differentiated	F	Alveolar ridge	46	NS
	16	Well differentiated	M	Mouth floor	58	S
	17	Well differentiated	M	Alveolar ridge	42	S
	18	Well differentiated	M	Lip	70	S
	19	Well differentiated	M	Alveolar ridge	66	S
	20	Moderately differentiated	M	Tongue	49	NS
	21	Moderately differentiated	M	Mouth floor	53	S
	22	Moderately differentiated	M	Tongue	51	S
	23	Moderately differentiated	M	Mouth floor	79	S
	24	Moderately differentiated	F	Alveolar ridge	85	S
	25	Moderately differentiated	M	Tongue	64	S
	26	Moderately differentiated	M	Tongue	74	S
	27	Moderately differentiated	M	Mouth floor	51	S
	28	Moderately differentiated	M	Buccal mucosa	74	NS
	29	Moderately differentiated	F	Alveolar Ridge	89	NS
	30	Moderately differentiated	M	Alveolar ridge	51	S
	31	Undifferentiated	M	Buccal mucosa	50	NS
	32	Undifferentiated	M	Alveolar ridge	67	S
	33	Undifferentiated	M	Alveolar ridge	38	S
	34	Undifferentiated	M	Mouth floor	49	S
	35	Undifferentiated	M	Alveolar ridge	68	NS
	36	Undifferentiated	M	Tongue	46	S

OED = oral epithelial dysplasia; CIS = carcinoma *in situ*; M = male; F = female; S = smoking; NS = non-smoking.

Table 2. Expression of laminin-332 γ2 chain and MMP-9

Expression	High risk oral lesion (n = 10)	OSCC (n = 26)	P-value
Laminin-332 γ2 in basement membrane			
Continuous	60% (n = 6)	0	0.000[a]
Discontinuous	40% (n = 4)	69.2% (n = 18)	
Absence	0	30.8% (n = 8)	
Laminin-332 γ2 in epithelium			
BL	10% (n = 1)	35% (n = 7)	0.2103
BL+SL	90% (n=9)	65% (n = 13)	
MMP-9 in epithelium			
BL	50% (n = 5)	35% (n = 7)	0.4072
SL	30% (n = 3)	20% (n = 4)	
BL+SL	20% (n = 2)	45% (n = 9)	
MMP-9 stroma/field			
< 25% cells/field	40% (n = 4)	11,5% (n = 3)	0.0086[a]
25 - 50% cells/field	50% (n = 5)	15,4% (n = 4)	
> 50% cells/field	10% (n = 1)	73.1% (n = 19)	

[a]Chi-square test (significance level P < 0.05).
OSCC = oral squamous cell carcinoma; MMP = matrix metalloproteinase; BL = basal layer; SL = suprabasal layer.

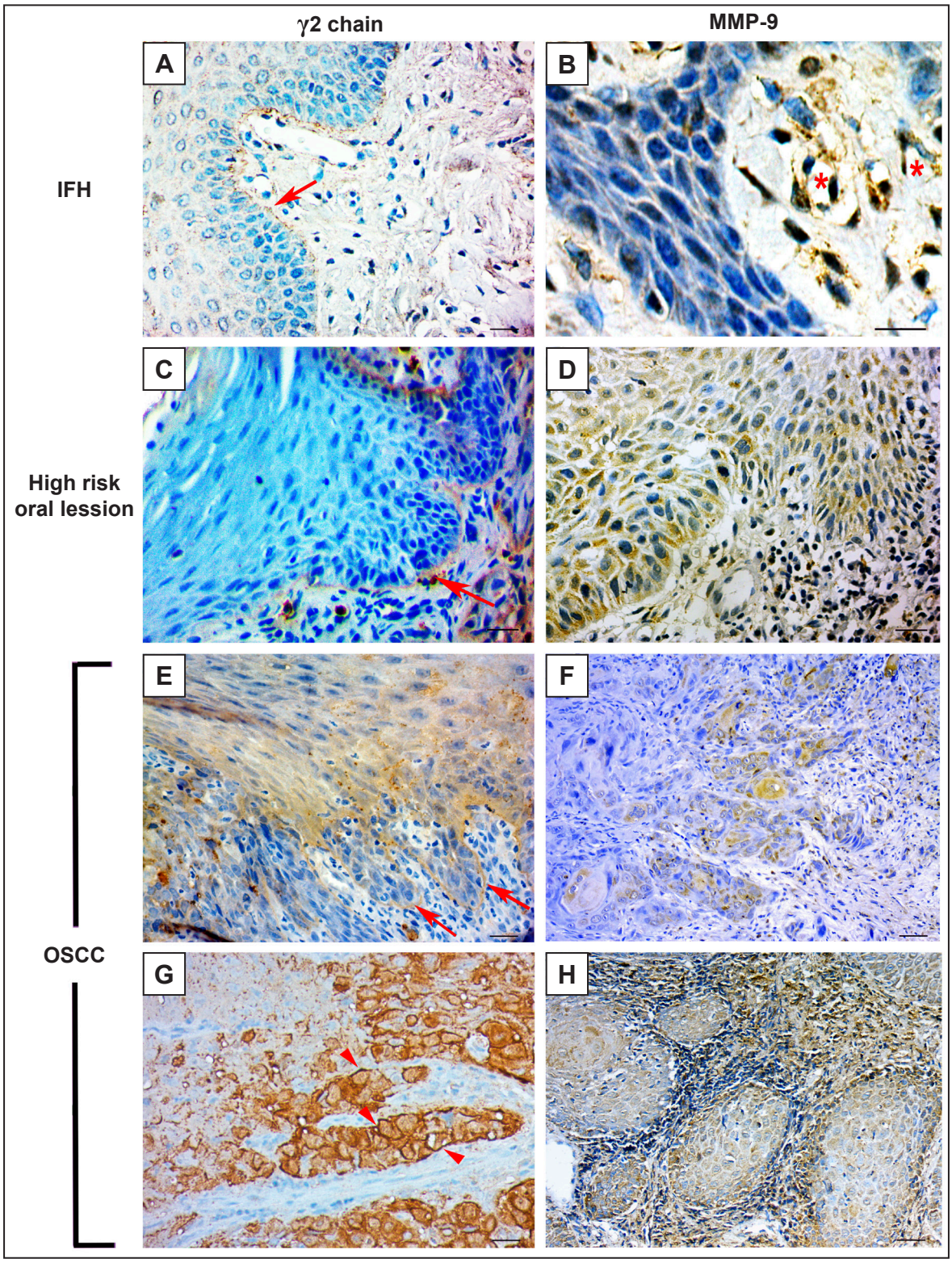

Figure 1. Expression of laminin-332 γ2 chain and MMP-9 in high risk of malignant transformation oral lesions and oral squamous cell carcinoma (OSCC) was examined by immunohistochemistry. Scale bar = 20 µm.
A = Positive control of laminin-332 γ2 chain along epithelial-stromal interface, showing a linear structure (arrow).
B = MMP-9 expression in endothelial cells of IFH (* symbol).
C = High risk oral lesion with continuous basement membrane (arrow).
D = MMP-9 citoplasmatic staining in epithelium cells.
E = OSCC well differentiated with areas of continuous basement membrane (arrows) and laminin-332 γ2 chain expression in SL.
F = Epithelium citoplasmatic expression of MMP-9 in OSCC.
G = Areas of invasive front with laminin-332 γ2 chain citoplasmatic expression and a cellular membrane staining in OSCC moderately differentiated (arrowhead).
H = Enzyme expression in stromal cells surrounding OSCC nests.
IFH = inflammatory fibrous hyperplasia; MMP = matrix metalloproteinase; BL = basal layer; SL = suprabasal layer.

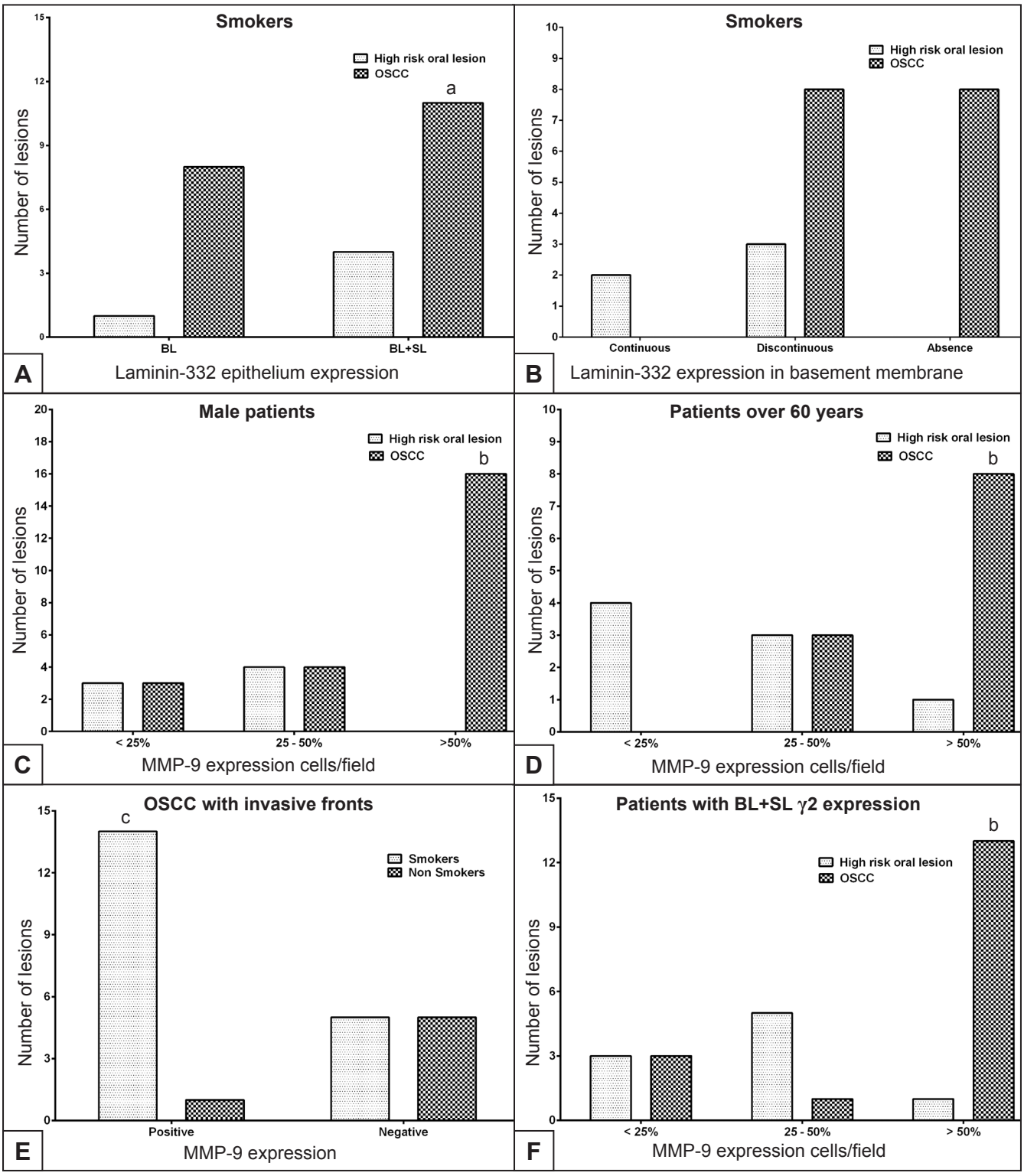

Figure 2. Graph analysis of association between clinical data and molecules expression.
A = Smokers with OSCC had a higher expression of laminin-332 γ2 chain in BL and BL+SL than high risk oral cases ($P < 0.0001$).
B = The same was not observed in basement membrane ($P > 0.05$).
C = Regarding MMP-9 expression, male patients with OSCC showed more stromal cells with citoplasmatic staining than high risk oral cases ($P = 0.0054$).
D = The same pattern was observed in patients older than 60 years ($P = 0.0101$).
E = The analysis of MMP-9 in invasive fronts demonstrated that smokers had a higher expression when compared with non-smokers ($P = 0.0225$).
F = Group of patients with laminin-332 expression in BL+SL with OSCC showed a higher frequency of stromal cells with citoplasmatic staining of MMP-9 than high risk oral lesions ($P = 0.003$).
[a]Two-way ANOVA (A and B), [b]Chi-square test (C, D and F) and [c]Fischer`s Exact test (E) (significance level $P < 0.05$).
OSCC = oral squamous cell carcinoma; MMP = matrix metalloproteinase; BL = basal layer; SL = suprabasal layer.

The analysis of MMP-9 in invasive fronts demonstrated that smokers had a higher (P = 0.0225) expression when compared with non-smokers (Figure 2E). Finally, when the group of patients with laminin-332 expression in BL+SL was evaluated, OSCC cases showed a higher frequency of stromal cells with citoplasmatic staining of MMP-9 than high risk oral lesions (P = 0.003) (Figure 2F).

DISCUSSION

The study sought to analyse molecular profile of laminin-332 γ2 chain and MMP-9 in high risk oral lesions and OSCC. In addition, microscopic features were evaluated in order to detected possible associations between histological and clinical factors. Our data demonstrated a discontinuous basement membrane and a higher expression of laminin-332 γ2 chain in epithelial compartment and invasive fronts related to smoking patients diagnosed with OSCC. Moreover, MMP-9 expression in stromal cells was prevalent in male.

Regarding clinical data, in high risk oral lesions individuals over 60 years were more prevalent and in OSCC cases the main age was over 50 years. In a retrospective study of 295 cases of OSCC, 81% of patients were men, with 51 - 60 years and the most prevalent site was the mandibular alveolar ridge [15]. The authors mentioned that cigarette consumption was the main habit that led to oral cancer. Within the context of high risk oral lesions, usually occurred in middle age men and prevalence increased considerably over 70 years [16].

A relevant point to improve cancer diagnostics is the development of biomarkers to detected cell early alterations. In this context, histopathological changes in basement membrane is a well-recognized step in epithelial cancer progression [7]. This process has been shown to be critical in carcinogenesis once tumour cells need a substrate to invade and proliferate [17]. We analysed laminin-332 γ2 chain expression in tissues of high risk oral lesions and OSCC. Study results demonstrated that most of high risk oral cases had a continuous basement membrane and any lesion was detected with a completely absent membrane. On the other hand, in OSCC lesions major of cases had a discontinuous or absence membrane. Those findings are in agreement with Imura et al. [18] that demonstrated a continuous and linear basement membrane while disease has not reached advanced stages.

In the connective tissue, OSCC showed γ2 expression in the periphery of tumour islands and in the tumour-stroma interface, especially in areas of invasive fronts. Zargaran et al. [19] demonstrated that γ2 chain expression was preferably detected in cells of invasive fronts and it was associated with tumour progression. These findings suggest that laminin-332 could play a role in the acquisition of a migrating profile, a feature that is required for malignancy.

When only smoking patients were considered, the association of clinical data and laminin-332 γ2 chain expression demonstrated a higher staining in epithelium of OSCC cases when compared with high risk oral lesions. Tobacco is a well-known risk factor in carcinogenesis and it was related to the acquisition of mobility in tumour cells [20]. The literature associated proliferation, migration and invasion activities with a profoundly change in γ2 chain. Garg et al. [21] showed a decrease in migration and invasion when cells were treated with silencing LAMγ2. Additionally, laminin-332 stimulates tumour cells to form fine protrusions on the sheet-shaped front edge membranes, which led to an increase in cell migration and invasion to the underlying tissue [22]. So, it is possible to suggest a role of laminin-332 γ2 chain in getting a more aggressive cancer phenotype.

The loss of basement membrane may be associated with stromal invasion and metastatic process. MMPs are involved in cleavage process of basement membrane. MMP-9 synthesis by tumour cells is important to those processes and could represent a feature of disease aggressiveness [9]. Our analysis demonstrated a higher cytoplasmic staining of stromal cells (more than 50% cells/field) in OSCC patients when compared to high risk oral lesions. Tamamura et al. [14] described few or no expression of MMP-9 in OED, which is in agreement with our results. Regarding correlation between clinical data and MMP-9 expression, in the cases of smoker patients with OSCC an elevated expression was observed in invasive fronts. It is important to consider that MMPs have the ability to modulate tumour invasion and its expression is well recognized in areas such as tumour fronts [14].

When only male patients over 60 years were analysed, those diagnosed with OSCC showed more stromal cells with cytoplasmic labelling of MMP-9 than high risk oral lesions. The increase in diffuse brownish granules of MMP-9 represented a more aggressive behaviour of disease in males and elderly patients. According to Georgescu et al. [23], MMP-9 expression in the stromal cells of colorectal carcinoma was related to the propensity for invasion and metastasis. The study also demonstrated a higher expression of MMP-9 in peritumoral lymph nodes, although metastasis-free, suggesting that

this enzyme is preparing tissue for a possible metastasis. Andisheh-Tadbir et al. [24] analysed 42 patients with OSCC and observed a statistical significance for tumour clinical stage. The authors found lymph nodes invasion in 100% of the samples with a high positive staining for MMP-9 in N1 stage. These data indicates aggressiveness of the OSCC related to MMP-9 expression.

Analysis of laminin-332 γ2 chain and MMP-9 expression in high risk oral lesions and OSCC may improve the understanding of different stages in oral carcinogenesis. Furthermore, seeking possible associations between molecules profile and clinical data may provide insight into how risk factors could modify tumour microenvironment.

CONCLUSIONS

Our results support the hypothesis of changes in molecules expression in high risk oral lesions and oral squamous cell carcinoma. A relation between clinical and molecule profile was observed. A discontinuous basement membrane and a higher expression of laminin-332 γ2 chain in epithelial compartment and invasive fronts was related to smoking patients diagnosed with oral squamous cell carcinoma. Moreover, matrix metalloproteinase-9 expression in stromal cells was prevalent in male over 60 years. However, other aspects may also participate of those changes and should be the subject of further studies.

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The authors declare no conflict of interest.

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