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Communication

Evaluation of the extent of interstitial fibrosis in oral squamous cell carcinoma compared with normal oral mucosa and oral epithelial dysplasia.

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Abstract: Objective: To evaluate the extent of interstitial fibrosis in samples of normal oral mucosa (NOM), oral epithelial dysplasia (OED) and oral squamous cell carcinoma (OSCC). Materials and method: Descriptive study. Eighteen samples of NOM, 15 samples of OED, and 13 samples of OSCC were analyzed; all stained with Masson's trichrome stain. The areas of greatest fibrosis underlying the normal, dysplastic, and malignant neoplastic oral epithelium were identified in order to determine the extent of interstitial fibrosis. Interstitial fibrosis was classified according to its proportion in the total image, being 0 (without fibrosis), +1 (1-25%), 2+ (26-50%), 3+ (51-75%) and +4 (76-100%). Variables were analyzed using the Kruskal-Wallis test and Dunn's Pairwise post-hoc test. Results: The samples of NOM and OED did not present interstitial fibrosis (type 0) in the majority of the cases respectively. OSCC samples were characterized by an extension of type 2+ interstitial fibrosis in 45% of all cases of OSCC. The extent of interstitial fibrosis was different between NOM and OSCC (p<0.001), and between OED and OSCC (p<0.001). Conclusion: The extent of interstitial fibrosis is directly proportional to the malignization of the analyzed samples, being an adequate marker for OSCC.

Keywords: Oral neoplasia; tumor microenvironment; fibrosis.

INTRODUCTION.

A malignant neoplasm has been defined as a cell mass surrounded by its microenvironment. It has been reported that the microenvironment of a tumor is formed mainly by irreversibly activated fibroblasts, known as cancer-associated fibroblasts. These cells synthesize growth factors that induce an increase in the synthesis of type I collagen, regulating interstitial fibrosis, influencing the growth, invasion and metastasis of tumors.

Cancer-associated fibroblasts are heterogeneous cells. Most of them derive from resident fibroblasts, although they can also be derived from mesenchymal and epithelial stem cells, pericytes, adipocytes, and endothelial cells.[1] These cells are thought to trigger proinflammatory and promoter functions that stimulate the development and progression of tumors during pathological remodeling in the early stages of the development of a malignant neoplasm.[2]

A study in an animal model conducted by Raimondi *et al.*,[3] determined that marked interstitial fibrosis develops at the beginning of oral carcinogenesis, even before the beginning of epithelial morphological changes that characterize dysplastic changes. Later, Lao *et al.*,[4] evaluated

the extent of interstitial fibrosis in dysplasia and spindle carcinoma in lingual samples, associating their marked presence with a low survival rate.

Therefore, the presence of interstitial fibrosis could be a marker of OSCC.

The aim of this study was to evaluate the extent of interstitial fibrosis in samples of normal oral mucosa (NOM), oral epithelial dysplasia (OED) and oral squamous cell carcinoma (OSCC), including different regions of the oral mucosa.

MATERIALS AND METHODS.

A descriptive study was carried out, approved by the Bioethics Committee of Universidad Andrés Bello (folio 002017.36). Eighteen samples with diagnosis of NOM, 15 with OED, and 13 with diagnosis of OSCC, obtained from the Histopathology Unit at the School of Dentistry at Universidad Andrés Bello, between 2004 and 2012, were included in the study.

Selection criteria

Paraffin embedded samples with adequate tissue to obtain 4-micron histological lamellae, histologically diagnosed with NOM, OED or OSCC, containing information about the age, sex of the patient and location of the diagnosis, were included in the study.

Samples with information related to OSCC due to recurrence or metastasis in the oral mucosa and samples with artifacts were excluded from the study.

Confirming diagnosis of the samples

Diagnosis confirmation of NOM, OED and OSCC was performed by two independent, previously standardized and calibrated pathologists who examined the samples under an optical microscope (Primo StarTrinocular, Zeiss®, Germany), in sections stained with hematoxylin-eosin.

Staining technique for Masson's Trichrome stain

Four-micron slices were obtained; they were deparaffinized and hydrated in distilled water. The sample was fixed in formaldehyde solution and with Bouin's fluid for 1 hour at 56-60°C. Then it was cooled and washed in distilled water.

The slice was stained with ferric hematoxylin for 10 minutes and washed in distilled water for 10 minutes. Subsequently, dyeing with acid scarlet-fuchsine solution was carried out for 2-5 minutes, and then the sample was washed with distilled water. It was treated with phosphotungstic acid solution for 10-15 minutes and was stained with aniline blue solution for 15 minutes. It was washed in distilled water, dehydrated, clarified and mounted on a slide.

Analysis of samples stained with Masson's Trichrome stain

The evaluation of the extension of interstitial fibrosis was performed by two independent oral pathologists, previously standardized and calibrated intra and inter-operator, under an optical microscope (Primo StarTrinocular, Zeiss®, Germany), at a 10X magnification, looking for areas of blue tonalities (Figure 1).

The extent of interstitial fibrosis was classified according to its proportion in the total image, being 0 (without fibrosis), +1 (1-25%), +2 (26-50%), +3 (51-75%) and +4 (76-100%).

Variables

Variables under study were age (years), sex, diagnosis (NOM-OED-OSCC), location (high risk: lateral border mucosa and lingual belly, and floor of mouth, low risk: mucosa in jugal, labial, palatal, alveolar, gingiva and lingual dorsum areas) and the extension of interstitial fibrosis (0, +1, +2, +3 and +4).

Statistical analysis

Qualitative variables were represented by absolute and relative frequency. The analysis of two categorical variables was performed by Fisher's exact test. The analysis of the age and extent of fibrosis according to diagnosis was made using the Kruskal-Wallis test. Conover-Iman post-hoc test was used to evaluate the differences in the ranges. A significance level of $p<0.05$ was used and statistical analysis was carried out with the STATA 12 program (StataCorp, Texas, USA).

RESULTS.

The number of samples included, the distribution by age and sex, and the extent of interstitial fibrosis according to the diagnosis NOM, OED and OSCC, are shown in Table 1.

The diagnoses of NOM, OED and OSCC were not associated with sex ($p= 0.483$) or to the location of the lesions ($p=0.114$). The extent of fibrosis was only different in the OSCC group with respect to NOM and OED ($p<0.001$).

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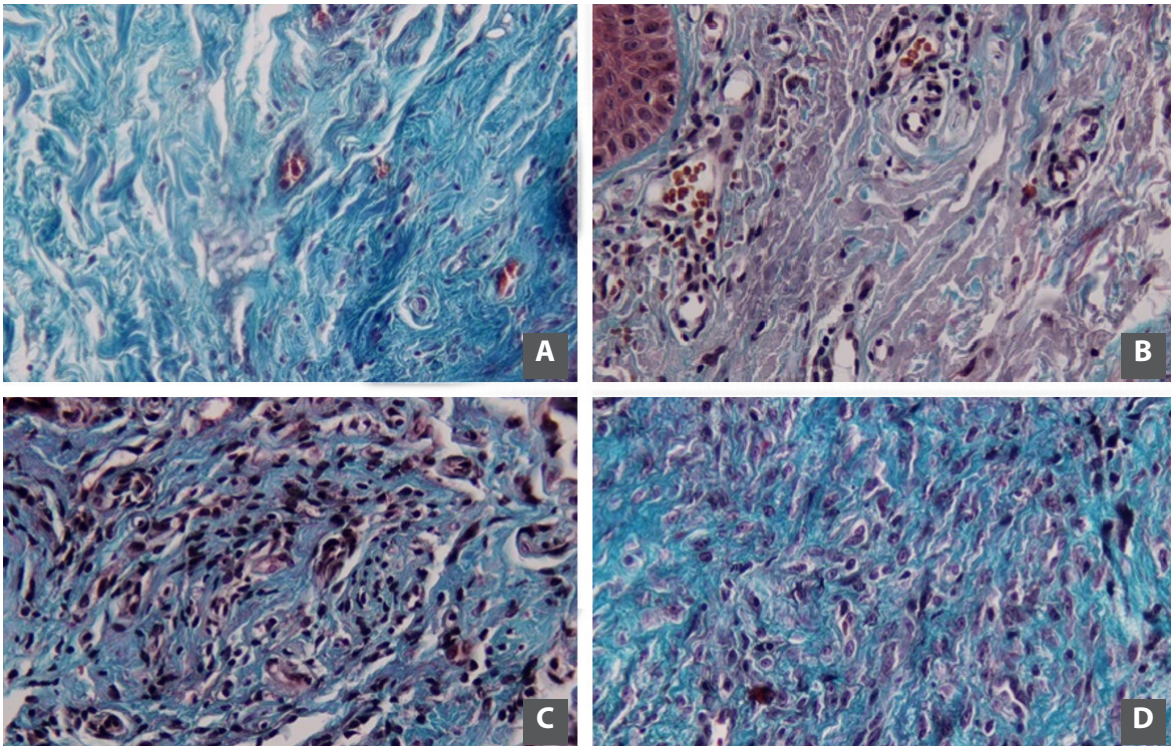
Figure 1. Histological samples stained with Masson's Trichrome stain.



A. Normal oral mucosa, extension +1 of insterstitial fibrosis. 40x. **B.** Mild dysplasia, extension +4 of insterstitial fibrosis. 40x. **C.** Well differentiated oral squamous cell carcinoma, extension +2 of insterstitial fibrosis. 40x. **D.** Moderately differentiated oral squamous cell carcinoma, extension +2 of insterstitial fibrosis. 40x.

Table 1. Description of the samples and extension of fibrosis, according to diagnosis.

		NOM	OED	OSCC
Number of samples.		18	15 (Slight OED 12, mild OED 2, severe OED 1)	13 (Well differentiated 4, moderately differentiated 9)
Mean age, years		21	56	75
Interquartile range, years		9 to 36	37 to 86	60 to 94
Sex	Males	5 (27.78%)	7 (46.67%)	6 (46.15%)
	Females	13 (72.22%)	8 (53.33%)	7 (53.85%)
Fibrosis, extension	+0	11 (61.1%)	11 (73.3%)	0 (0%)
	+1	66 (33.3 %)	2 (13.3%)	4 (30.7%)
	+2	0 (0%)	1 (6.67%)	6 (45.15%)
	+3	1 (5.56%)	0 (0%)	2 (15.38%)
	+4	0 (0%)	1 (6.67%)	1 (7.69%)

DISCUSSION.

The mean age was 56 and 75 years for the groups diagnosed with OED and OSCC, respectively. These results agree with what has been reported in the Chilean and international literature.[5,6]

No significant differences were found by sex according to the diagnosis of OED and OSCC. However, Martínez *et al.*[5] reported that in Chile OED is higher in women and OSCC is higher in men. These differences could be due to the insufficient epidemiological information on individuals diagnosed with OED and OSCC in Chile.[5]

The tongue and floor of the mouth are considered the most frequently affected areas by OED and OSCC. They are known as high risk areas as they are covered by a thin, non-keratinized epithelium directly exposed to carcinogenic agents such as tobacco and alcohol.[5] However, in this study, no difference was found in the location of OED and OSCC in areas of high and low risk, which coincides with what was published by Riera and Martínez.[7] This result could be attributed to the fact that OED and OSCC are not only

associated with tobacco and alcohol consumption, but also with genetic, immunological and dietary factors, among others, which could also play a significant role.[8]

The majority of the NOM samples did not present interstitial fibrosis, however, 33% of them presented interstitial fibrosis type 1, and some reached the extension of type 3. This can be attributed to the fact that fibrosis would play a role in the maintenance of the structure of the oral mucosa.[3] A high percentage of OED did not present interstitial fibrosis, however, some samples reached type extension 1+, 2+ and even 4+. This can be explained by the fact that most of the OED evaluated in this study corresponded to mild OED and that the samples that showed a marked extension of fibrosis may be indicating the beginning of the progression to carcinoma.[3]

On the other hand, interstitial fibrosis was identified in all samples of OSCC, with type 2+ being the most frequent. The extent of interstitial fibrosis was statistically significant between the OSCC/NOM and OSCC/OED groups, which coincides with that reported by Lao *et al.*,[4] These results can be attributed to the fact that the recruitment and the irreversible activation of fibroblasts, metabolically active to synthesize collagen, occurs in the OSCC stage and not in previous stages of NOM or OED.[4,2,9]

In addition, it is important to note that fibroblasts that are irreversibly activated in cancer present key functions for tumor growth and metastasis. These functions include the regulation of metalloproteinases, angiogenesis, lymphogenesis and the immune system.[2,10]

CONCLUSION.

The extent of interstitial fibrosis is directly proportional to the malignization of the analyzed samples, being an adequate marker for OSCC.

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