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Article

Difference in EGFR expression and mean vascular density in normal oral mucosa, oral epithelial dysplasia and oral squamous cell carcinoma.

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Abstract: To evaluate the expression of the epidermal growth factor receptor (EGFR) and mean vascular density (MVD) in normal oral mucosa (NOM), oral epithelial dysplasia (OED) and oral squamous cell carcinoma (OSCC). Material and methods: Descriptive case study. Nineteen histological samples diagnosed with NOM, 18 diagnosed with OED, and 19 with OSCC, were analyzed with immunohistochemistry against EGFR and CD31. EGFR expression was evaluated by extent and intensity of its expression in normal, dysplastic and neoplastic epithelium. MVD was determined through the detection of blood vessels by antibodies against CD31. Results: Extension of EGFR expression was highest in OSCC followed by OED and lowest in NOM, resulting in significant different between the degrees of extension (p<0.001). Intensity of EGFR was similar in NOM, OED and OSCC, without differences in its expression (p=0.533). Differences in MVD were found between NOM and OSCC groups (p<0.01), and between OED and OSCC groups (p<0.01), with no differences between NOM and OED groups (p=0.91). MVD was 21.17±4.98 in NOM, 23.40±5.77 in OED and 33.92±8.39 in OSCC. Conclusion: EGFR is expressed in normal, dysplastic or neoplastic oral epithelium. However, the extent of its expression is greater as malignancy increases. MVD varies according to the diagnosis.

Keywords: Oral cavity, mouth neoplasms, epidermal growth factor receptor, pathologic angiogenesis.

INTRODUCTION.

Oral squamous cell carcinoma (OSCC) is the most common malignant neoplasm of the oral cavity. [1] An incidence of 500,000 cases per year has been reported worldwide.[2] OSCC may originate from malignant transformation of normal oral mucosa (NOM), and from potentially malignant lesions with different degrees of oral epithelial dysplasia (OED).[3,4]

The genesis of malignant neoplasms, such as OSCC, is a complex process involving a breakdown in the regulation pathways of cell division, differentiation, death and angiogenesis.[2] In this regard, protooncogenes are the physiological regulators of proliferation and differentiation of normal cells. Overexpression of their mutated counterparts, the oncogenes, plays a key role in carcinogenesis. Oncogenes encode, among others, growth factor receptors, which may cause uncontrolled cell proliferation. In the case of carcinomas, one key receptor is the epidermal growth factor receptor (EGFR).[5] Its mutation stimulates mitosis and inhibits the apoptosis of neoplastic keratinocytes.[6] This receptor is expressed both in normal epithelia and in those subjects with pathologies such as epithelial dysplasia and carcinomas, where proliferation and differentiation of

keratinocytes are altered.[7]

The complex interaction between neoplastic cells and their environment plays a central role in carcinogenesis, as environmental changes may facilitate cell growth, invasion and metastasis. In the case of OSCC, the microenvironment consists of fibroblasts, deposits of the extracellular matrix, immune system cells, lymphatic vessels and blood vessels. The vascular system supplies oxygen and nutrients to the neoplastic cells. In addition, newly formed endothelial cells secrete growth factors that act on themselves and on adjacent neoplastic cells stimulating their proliferation.[8,9] Malignant neoplasms induce angiogenesis in a volume up to 2-3 mm^3 and this value represents the critical distance by which nutrients and oxygen can diffuse from the blood vessels.[10] Therefore, the understanding of angiogenesis is critical to comprehend the malignant transformation of epithelial lesions of the oral cavity such as OED or OSCC.[11,12]

CD31 is one of the most important molecular markers for evaluating angiogenesis, through the calculation of the mean vascular density (MVD). CD31 is a protein present in the intercellular junction of endothelial cells in developing or already developed blood vessels.[13]

The aim of this study was to evaluate EGFR expression and angiogenesis, through the mean vascular density (MVD), in NOM, OED and OSCC.

MATERIALS AND METHODS.

A descriptive case study was designed. The study was approved by the Bioethics Committee of Universidad Andrés Bello (Folio No 033). Participants were asked to sign an informed consent.

Sample collection

Nineteen samples diagnosed with NOM, 18 diagnosed with OED, and 19 with OSCC, were collected. Samples of NOM were obtained from alveolar ridge mucosa of mandibular third molars, from individuals who had undergone surgery at the School of Dentistry of Universidad Andrés Bello, Viña del Mar, between March and July 2014. Samples of OED and OSCC were obtained from paraffin-embedded samples collected between 2004 and 2012 by the Oral Histopathology Service at the School of Dentistry, Universidad Andrés Bello, Viña del Mar.

Inclusion and exclusion criteria for sample selection

Inclusion criteria included paraffin-embedded samples with enough tissue to obtain three histological section of 4 microns each, diagnosed histologically with NOM, OED or OSCC, with information regarding the patient's age, gender and place of residence. All age ranges, both sexes and location of the lesion in the oral mucosa were included. Exclusion criteria consisted of OSCC samples from secondary to metastasis stage and histological lamellae with methodological artifacts.

Confirming diagnosis of the samples

To confirm the diagnosis of NOM, OED and OSCC of the selected samples, two independent, previously standardized and calibrated pathologists examined hematoxylin-eosin stained sections under Olympus® CX-31 light microscopy (Olympus Corporation, Japan). In order to make the diagnosis, they considered the criteria proposed by the World Health Organization in a double-blind examination.[14,15]

Immunohistochemistry technique for EGFR

Four micron sections were obtained and mounted on xylanized, dewaxed slides and hydrated with distilled water. Once hydrated, an antigenic recovery process was performed on a steamer using citrate buffer, pH 6. The endogenous peroxidase enzyme was then blocked by the application of 3% v/v hydrogen peroxide. Sections were incubated overnight with rabbit anti-EGFR monoclonal antibody (diluted 1: 100; Ventana Medical System Inc, Tucson Arizona, USA). Immunostaining was performed with Envision system (Dako, Santa Clara, USA.) according to the manufacturer's instructions. Peroxidase activity was measured by the application of the diaminobenzidine chromogen substrate.

Immunohistochemistry technique for CD31

Samples were processed as described for the immunohistochemistry of EGFR, but were placed on a poly-L-lysine coated slide (BioSB, Santa Barbara, USA). These sections were incubated with human CD-31 monoclonal antibody (Clone JC70A, IgG-1, kappa, Dako, Carpenteria, California, USA), diluted 1:40, using the avidin-biotin-peroxidase complex detection method, at a temperature of 37°C for 32 minutes. Antigenic recovery was performed at 95-100°C for 60 minutes with CC1 Standard solution (Cell Conditioning Solution-1, Ventana Medical Systems, Inc.).

Evaluation of immunostained samples against EGFR

Samples were analyzed by two blind calibrated examiners (RM and AF). Immunopositive cells for EGFR were those that presented membrane and/or cytoplas-

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mic staining, compared with negative and positive controls placed on the same slide. The positive control consisted of a segment of placenta analyzed with the complete immunohistochemical technique for EGFR. The negative control was obtained by omission of the primary antibody.

The intensity of immunostaining of EGFR in NOM, OED and OSCC was qualitatively evaluated and categorized nominally and arbitrarily into 0: negative immunostaining, 1: mild immunostaining, 2: moderate immunostaining and 3: marked immunostaining. The extent of EGFR immunostaining in the epithelial thickness of NOM, OED and OSCC was categorized as 0=0%; 1=1 to 25%; 2=26-50%; 3=51-75%; 4=75-100%.

Evaluation of immunostimulated samples against CD31

Samples were analyzed by two blind calibrated examiners (RM and AF). Any tubular structure coated by endothelial cells, individual endothelial cells or in islets immunoreactive with the antibody against CD31, was considered as a blood vessel. Each sample was compared with a positive and negative control in each histological slides. The positive control consisted of a segment of angiosarcoma stained with the complete immunohistochemical technique against CD31. The immunonegative control was obtained by omission of the primary antibody.

To determine MVD, samples were observed under an Olympus® CX-31 light microscope (Olympus Corporation, Japan), and 3 consecutive hot spots were selected that corresponded to the areas of greatest vascularization. Each hot spot was photographed at 40x magnification, using a 5.1 megapixel Micrometrics® Model 518 CU digital camera built into the microscope. In each image blood vessels were counted (20x objective lens and 10x ocular lens, 0.7386 mm^2/field).

MVD of each sample was determined using the following formula: MVD=(Number of vessels in Hot spot 1+ Number of vessels in Hot spot 3 + No of Hot spot 3 vessels)/3.

Statistical analysis.

Parametric and non-parametric tests were performed according to the nature of the variables. Kruskal-Wallis test was used for the analysis of the extent and intensity of EGFR according to diagnosis, and Conover-Iman post-hoc test was performed to evaluate differences in the range averages. The comparison of the mean number of vessels according to diagnosis was performed using ANOVA, with Bonferroni post hoc test. Student's t-test of independent samples was used to evaluate the relationship between EGFR extension and the number of vessels. Statistical significance was set at $p<0.05$. Statistical analysis was performed with STATA 12® (StataCorpLP, Texas, USA).

RESULTS.

The number of samples included in the study, age and gender distribution, for each diagnosis and the totality of the samples, are shown in Table 1.

Regarding EGFR expression by extension and intensity (Figure 1, Table 2), there was a difference in the extent of EGFR expression when comparing each diagnostic group ($p<0.001$). In contrast, no differences were found in the intensity of detected EGFR when comparing each diagnostic group ($p=0.533$). On the other hand, when analyzing OSCC samples according to their degree of differentiation,

Table 1. Distribution of patients by their demographic variables and clinical characteristics.

	NOM	OED	OSCC	Total sample
Number of samples	19	18	18	56
Median age (years)	22	56	73	55.5
Interquartile range (years)	17 to 25	49 to 65	65 to 82	25 to 65.7
Frequency of females %	73.68	26.32	50	50
Frequency of males %	57.89	42.11	60.71	39.2

NOM: normal oral mucosa; **OED:** oral epithelial dysplasia; **OSCC:** Oral squamous cell carcinoma.

Table 1. Comparison of the extent and intensity of EGFR expression in normal oral mucosa, oral epithelial dysplasia and oral squamous cell carcinoma.

	Extension						Intensity					
	2		3		4		1		2		3	
	26-50%		51-75%		476-100%		Mild		Moderate		Marked	
	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)
NOM	1	(5.26)	18	(94.74)	-	-	-	-	11	(57.89)	8	(42.11)
OED	-	-	14	(77.78)	4	(22.22)	1	(5.56)	12	(55.56)	7	(38.89)
OSCC	-	-	2	(10.53)	17	(89.17)	1	(5.26)	7	(36.84)	11	(57.89)

NOM: normal oral mucosa; **OED:** oral epithelial dysplasia; **OSCC:** Oral squamous cell carcinoma.

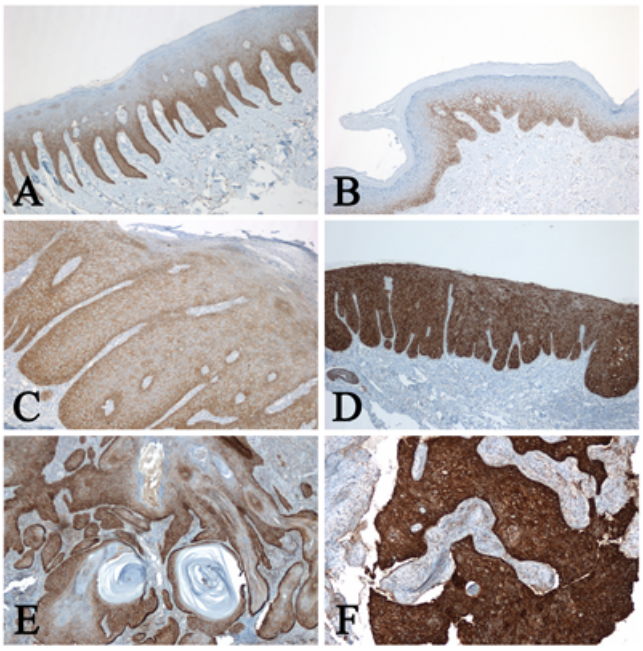

Figure 1. EGFR expression (Brown) in normal oral mucosa **(A)** and, oral epithelial dysplasia **(B:)** mild **(C)** and marked **(D)**. Oral squamous cell carcinoma: well differentiated **(E)** and poorly differentiated **(F)**. 10x magnification.

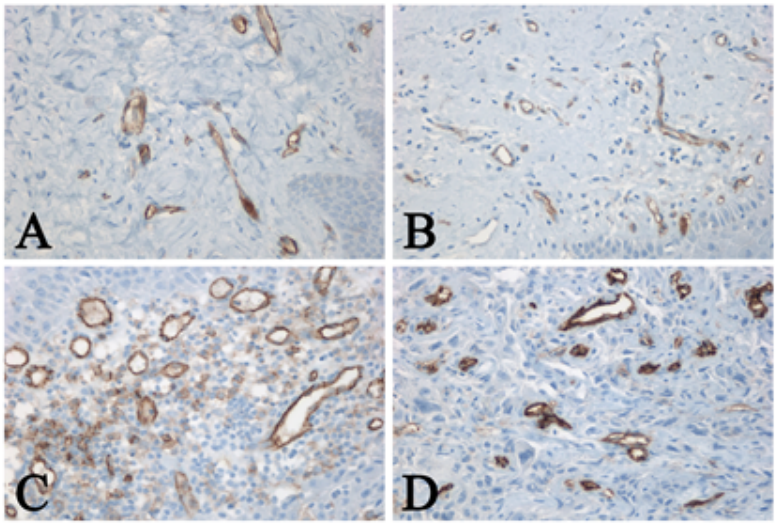

Figure 2. Expression (Brown) of CD31 showing blood vessels in normal oral mucosa **(A)**, mild oral epithelial dysplasia **(B)**, and oral squamous cell carcinoma **(C)** and **(D)**. 10x magnification.

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no differences were found in the intensity and extent of EGFR (p=0.704 and p=0.816, respectively).

Regarding MVD (Figure 2), a mean of 21.17±4.98 vessels were observed in NOM, 23.40±5.77 vessels in OED, and 33.92±8.39 vessels in OSCC. The MVD was different between NOM and OSCC (p<0.01) and between OED and OSCC (p<0.01). However, no differences were found between NOM and OED groups (p=0.91).

By relating MVD and the extent of EGFR expression in categories 3 and 4, at category 3 of EGFR expression there was a mean of 22.79±6.65 vessels, and at category 4 there was a mean of 31.55±8.83 vessels (p<0.001). In contrast, no differences were found between the number of vessels and the EGFR expression (p=0.351).

DISCUSSION.

In the last decade, interest has increased in identifying markers that may allow prediction of malignant transformation of normal and dysplastic oral epithelium into an OSCC.[7] This is due to the fact that even in spite of advances in scientific knowledge, the histopathological diagnosis remains the gold standard for making diagnostic and therapeutic decisions, which does not allow the prediction of how an oral lesion will evolve.[22]

It was found that dysplastic lesions of the oral mucosa preceded the neoplastic epithelial pathology, which coincides with that reported in the literature.[23,24] This may be because the pathogenesis of both OED and OSCC is associated with the same carcinogenic stimuli. However, in OSCC, it is associated with a long accumulation of its effects.[25,26] In the OED group, no gender bias was found. However, a higher frequency of OSCC was found among women. There is controversy as to which sex is most affected. This could be explained by region specific habits, such as chewing tobacco or betel nut.[23-26,27]

Regarding evaluation of EGFR, authors such as Rössle *et al.*[6] and Rajesweri *et al.*[28] and the authors of this study found that in all the samples analyzed, regardless of diagnosis, immunostaining of EGFR was always positive. However, there were variations in its intensity range. The extension of EGFR expression in the NOM group was mainly of type 3, reflecting that EGFR was expressed more in the basal, suprabasal and spinosum strata, disappearing in the superficial stratum, which coincides with that described in the literature.[28,29] The intensity of EGFR was mainly moderate and marked, which coincides with Rajesweri *et al.*[28] These results can be explained because EGFR is expressed in proliferating epithelial cells, such as in the basal stratum, and its expression is lost in the superficial or corneous stratum where the prevalence of proliferative cells decrease.[17] Unlike the NOM group, EGFR expression in the OED group in some samples involved even the stratum corrneum, which may be due to an increase in normal proliferative activity, although in an irregular growth pattern.[28] Its intensity was categorized as "marked", similarly to that of the NOM group, coinciding with the data reported in the literature.[29] In the OSCC samples, it was found that in most cases the extension of EGFR covered the entire sample and its intensity was defined as "marked" in a high percentage. Rössle *et al.*[6] and Sarkis *et al.*[8] reported similar findings. From this data, it could be inferred that the extent and intensity found for EGFR in carcinomas would be revealing a completely uncontrolled growth of neoplastic epithelial cells[12] and that the extent of EGFR expression is higher as malignancy increases.

In the present study, no difference was found between intensity and extent of EGFR regarding different degrees of OSCC differentiation, coinciding with Ragomir *et al.*[30] This fact may indicate that EGFR is not related to the degree of differentiation of neoplastic keratinocytes. Although Sarkis *et al.*[12] and Laimer *et al.*,[20] consider that both the extent and intensity of EGFR reflect an alteration in the regulation of cell proliferation. The results of this study suggest that the alteration of cell proliferation would be mainly represented by the number of cells affected and not by expression intensity.

In the 1970s the growth of tumors was associated with angiogenesis for the first time. The most important pro-angiogenic factor is VEGF and its activation may be positively regulated by EGFR.[31] We found that MVD showed a tendency to increase from NOM to OSCC. This difference was significant when comparing MVD of NOM and OSCC diagnoses and the diagnosis of OED and OSCC. This suggests that the angiogenic phenotypic change could occur mainly in OSCC rather than OED.[9] Other authors such as Sathyakumar *et al.*[32] Basnaker *et al.*[33] also studied MVD in samples with NOM, OED and OSCC diagnoses,

but used different types of markers for blood vessels. However, it should be noted that most of the studies, independent of the marker used, coincide with the results of this study in relation to the increase in MDV in the transition from NOM to OED to OSCC.

To our knowledge, the association between EGFR and CD[31] expression has not been previously studied in oral samples of NOM, OED and OSCC. This study demonstrates that when the EGFR extent was greater than 50%, independent of its diagnosis, MDV increased. These results suggest that EGFR could positively regulate angiogenesis, probably via secretion of growth factors. a recent publication showed that a decrease in EGFR was involved in the decrease of VEGF expression and that the activation of EGFR-VEGF favored angiogenesis in hepatocellular carcinoma.[34]

Some consideration should be given to the limitations of this study, such as sample size, due to the fact that OSCC is a low prevalence pathology[1] and the large difference in the age range of subjects in the NOM group, when compared to subjects in the OED and OSCC groups. The latter could be explained by the samples of NOM, which were obtained from mucosa that included third molars.

CONCLUSION.

EGFR is expressed in normal, dysplastic or neoplastic oral epithelium. However, the extent of its expression is greater as malignancy increases. MVD varies according to the diagnosis.

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