ORIGINAL RESEARCH

Epidermal Growth Factor Receptor (EGFR) is Overexpressed in Both Dysplasia and Neoplastic Oral Diseases.

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Abstract

Introduction: The Epidermal Growth Factor Receptor (EGFR) is a transmembrane cytoplasmic protein; studies indicate that it contributes to the development and progression of potentially malignant lesions to oral squamous cell carcinoma (OSCC). In the present study we determine and characterize the expression of EGFR in samples of oral epithelial dysplasia (OED) and OSCC.

Materials and Methods: 54 samples with histopathological diagnosis of OED were selected, 19 of low grade dysplasia (LGD) and 35 of high grade dysplasia (HGD) and a tissue matrix was with 60 samples of OSCC and 9 of normal oral mucosa were used. EGFR detection was performed by immunohistochemical technique. In the photomicrographs, protein expression was determined. Chi square test and Fisher test were used for statistical analysis (P <0.05).

Results: 54% of OED and 60% of OSCC showed a significant pattern of high EGFR expression when compared with normal oral mucosa.

Discussion: We found a tendency in LGD, HGD and OSCC to over expression of EGFR.

Conclusion: There is over expression of EGFR in OED and OSCC, so it could be considered as an early marker of these pathologies.

Key-words: Oral Epithelial dysplasia, Oral squamous cell carcinoma, EGFR.

Oral and Maxillofacial Pathology Journal (2022): ***https://www.ompj.org/archives.***

Introduction

Oral cancer originates as a sequential process, with accumulation of genetic, epigenetic, metabolic, hormonal and other consecutive alterations of exposure to carcinogens[1]. Oral squamous cell carcinoma (OSCC) is the most common malignant neoplasm of the oral cavity, accounting for 90% of oral cancers. Although OSCC may originate de novo, it is recognized that oral potentially malignant disorders (OPMDs) represent an increased risk of developing oral cancer, anywhere in the oral cavity[2-4].

Normally, the OPMDs most frequently associated with the development of OSCC are leukoplakia, erythroplakia and actinic cheilitis. The risk of malignant transformation of these lesions is mainly associated with the presence of a combination of cellular and architectural alterations, caused by the accumulation of genetic changes, known as oral epithelial dysplasia (OED)[1,5].

Although the degree of dysplasia evaluated by histological examination is currently the standard predictor of malignant transformation, it has significant limitations. Studies indicate that the risk of malignant progression varies between 6% and 36% in the presence of OED[6], and a higher probability of malignant transformation has been associated with lesions with a high degree of OED[3,7]. However, the evidence from longitudinal studies

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How to cite this article: Adorno-FariasD, Badilla SM, Vidal GP, Fernandez-RamiresR. Epidermal growth factor receptor (EGFR) is overexpressed in both dysplastic and neoplastic oral diseases. Oral MaxillofacPathol J 2022; 13(2): page no. 73-78.

Conflict of Interest: None

Ethical approval: All procedures performed in this study were in accordance with the ethical standards of the University of Chile (Approval No. 2014/29) committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The patients weren´t direct involved in this study.

Funding obtained: Ricardo Fernández-Ramires (FDP U Mayor PEP I-2022016, and FONDECYT 11140281) Daniela Adorno Farias (FIOUCH 2019 S19-14)

remains contradictory[8], there are documented cases of malignant transformation of mild dysplasia and complete regression of severe dysplasia, exposing the insufficiency of current risk classification schemes for OPMD[3,9-12].

Several biomarkers have been reported that can be used to predict aggressiveness, recurrence and resistance to conventional treatments in OSCC, representing a useful tool, especially in early tumor detection. Epidermal growth factor receptor (EGFR) is a transmembrane cytoplasmic protein that belongs to the ErbB family of receptor tyrosine kinases (RTK) encoded by the *EGFR* gene located on chromosome 7p12-13. EGFR activity plays an important role in the morphogenesis of organs derived from mesoderm and ectoderm, such as the brain, heart and lung. In the adult body, however, it loses this role. RTK are involved in the development of mammary ducts at puberty, the proliferation of the alveolar lobe in pregnancy and the production of milk in the postpartum period.[13]

EGFR has been found to be over expressed in various neoplasms and this overexpression has been correlated with a worse prognosis, higher proliferation rate, greater invasiveness and reduced survival[14-17]. Furthermore, an increase in the number of copies of the *EGFR* gene would be associated with the progression from OPMD to OSCC. Likewise, it is suggested that EGFR inhibitors can prevent oral cancer in patients with OPMD who have higher *EGFR* copy numbers[18,19]. This therapeutic option could be very useful for OPMD, avoiding aggressive surgical treatments, reducing recurrences and progression to OSCC.

Based on this background and knowing that there are only few studies in the literature that evaluate EGFR expression in relation to the different degrees of OED, this study aimed to determine and characterize EGFR in OED of different grades, comparing it with the expression in normal mucosa and OSCC in its four stages.

Materials and Methods

All procedures performed in this study were in accordance with the ethical standards of the University of Chile (Approval No. 2014/29; FONDECYT No. 11140281) committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The patients were not direct involved in this study. Fifty-four samples with a diagnosis of OED in different degrees were selected, obtained from the Pathological Anatomy Laboratory of the Faculty of Dentistry of the University of Chile. All histological slides stained with hematoxylin/eosin were reviewed by two examiners under a light microscope and classified according to the degree of OED in low (LGD) or high grade (HGD), in agreement with the WHO classification (2017)[1,20]. Clinical data on gender, age and anatomical location were obtained from anatomopathological records. In addition, 60 cases of OSCC, classified according to WHO 2017[1], and 9 cases of normal mucosa (NM) were used from the tissue microarray (TMA) model OR208 (Biomax Inc, 2016, USA).

For immunohistochemistry, 3µm sections were cut from formalin-fixed and paraffin-embedded material and mounted on silanized glass slides. Then, antigen retrieval was carried out in a pressure cooker (Oster®) with citrate buffer at pH 6 for 44 min, and endogenous tissue peroxidase was blocked in a 3% hydrogen peroxide solution and distilled water for 10 min, protected from light. Then, a 2.5% Normal Horse Serum Blocking Solution (Vector Laboratories, Burlingame, USA) was used for protein blocking at room temperature. Sections were incubated with the following monoclonal primary antibody: Anti-Rabbit EGFR (1:30; clone D38B1; Cell Signaling Technology)diluted in sterile phosphate-buffered saline (PBS), overnight at 4°C. Primary antibodies were replaced with PBS as a negative control in all reactions. As secondary antibody, R.T.U Biotinylated Goat Anti-Rabbit IgG (Vector Laboratories, Burlingame,

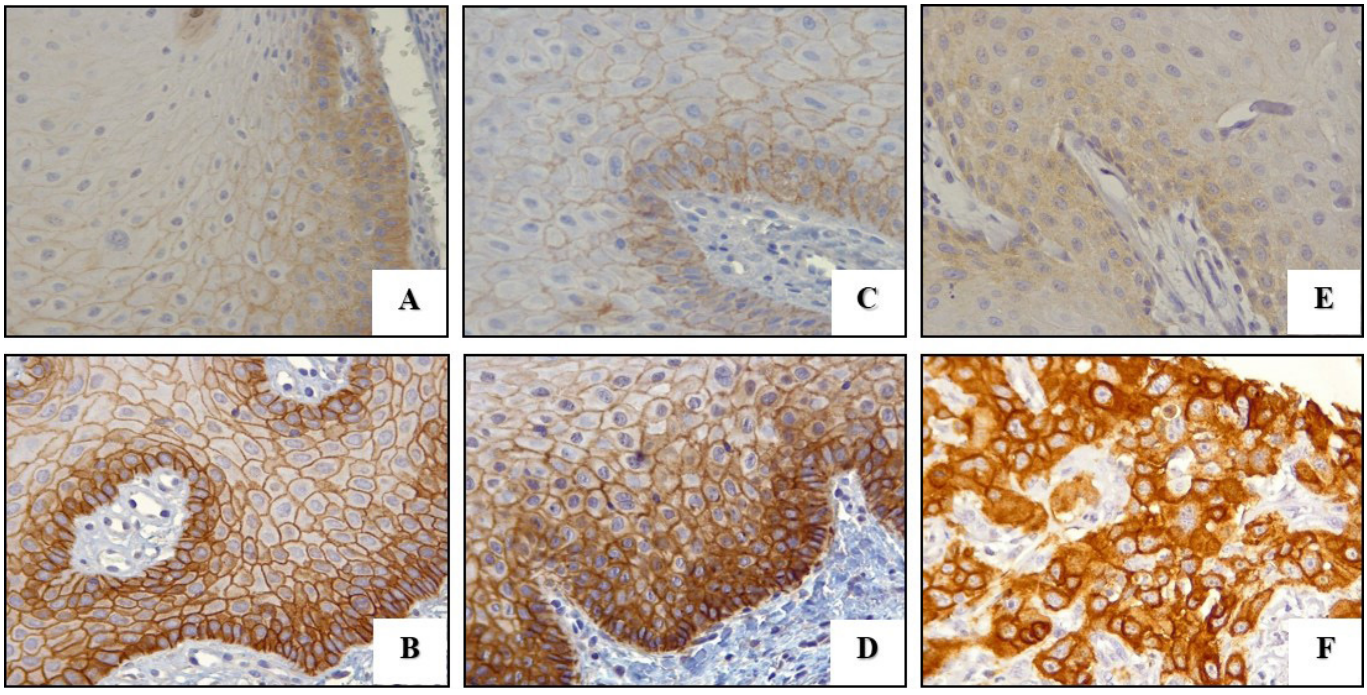

Fig. 1: Representative fields of LGD, HGD and OSCC with immunohistochemical staining of EGFR antibody. a) LGD with low EGFR expression; b) LGD with high EGFR expression; c) HGD with low EGFR expression; d) HGD with high EGFR expression; e) OSCC with low EGFR expression; f) OSCC with high EGFR expression.

USA) was used at 37°C. The reaction was revealed with 3,3'-diaminobenzidine (Dako Corporation, Carpinteria, USA) for 5 min in a dark chamber. The tissue samples were counterstained with Harris hematoxylin for 5 min, dehydrated and mounted. Mammary carcinomas were used as positive control.

Immunohistochemical staining of the EGFR antibody was analyzed by two observers independently, who were blinded to clinicopathological and immunohistochemical data. For this purpose, a high definition light microscope at 40x magnification (Leica DM750, Leica Microsystems, Switzerland, 2015) was used in ten consecutive fields, and the images were transferred to a video monitor using a computerized system. After capture with a digital camera (Leica ICC50 W, Leica Microsystems, Switzerland, 2015), the cases were analyzed using Image J software (NIH Image, USA, 2016). Cases exhibiting brown staining were defined as positives and labeling was scored to membrane location. Antibodies were scored according to labeling intensity and ratio. Intensity was scored as follows: negative staining = 0, weak staining = 1, moderate staining = 2, and intense staining = 3. The labeling ratio was defined as the percentage of labeled cells (0= 0–10 %, 1= 11–25 %, 2= 26– 50 %, 3= 51–75 %, 4= 76–100 %). The labeling index (LI) was calculated by multiplying the intensity (0–3) by the labeling ratio (from 0 to 4). Protein expression was classified according to the labeling index (LI) as follows: negative (-) for LI = 0, low expression (1+) for LI from 1 to 4, moderate expression (2+) for LI from 5 to 8 and high expression (3+) for LI ≥ 9 (21,22). Then, to compare immunolabeling between all groups, the LIs were classified into two groups, low expression (0 and 1+) and high expression (2+ and 3+) (Figure 1).

Differences in EGFR expression were analyzed using the chi-square test and Fisher's exact test. Significant differences were considered to exist if p-value< 0.05. All statistical calculations were performed using GraphPad Prism 6.03 software (San Diego, CA, USA).

Results

Of a total of 54 samples diagnosed as OED, 35 (64.8%) corresponded to LGD and 19 (35.2%) to HGD, with the mean age of LGD and HGD being 49 and 56 years, respectively. OSCC samples were separated according to their clinical stage, with 24 samples (40%) in stage 1, 16 (26.7%) in stage 2, 6 (10%) in stage 3 and 14 (23.3%) in stage 4. The results of the analysis of the clinical data of the samples are systematized in Table 1. The averages of ages and the number of female and male patients were similar in the OED and normal oral mucosa groups (p> 0.05).In the OSCC samples, the percentage of men was higher than that of women (p <0.05).

Table 2 shows the results of EGFR expression where most of the OED and OSCC samples in their different degrees, presented a significant pattern of high EGFR expression (p <0.05). On the other hand, the total NM samples presented homogeneous low expression of this marker. Stage 3 and 4 of OSCC showed a higher frequency of cases with high EGFR expression than stage 1 (p <0.00001). High EGFR expression was observed in only a few cases of stage 1 OSCC (p <0.00001).

Regardless of the grade, a greater number of OED cases positive for moderate to intense EGFR expression were found in the epithelial cells located in the basal and parabasal strata (Table 3). In addition, a statistically significant association was observed between high EGFR expression and moderate to intense staining in the basal and suprabasal strata (p <0.05). Table 4 shows that no association was found between EGFR expression and age, location or gender in patients with OED.

Discussion

EGFR overexpression occurs in around 80-90% of head and neck cancers[23] and has also been reported in oral premalignant lesions[19,22,24]. Furthermore, this pathway is of interest, as there are molecular therapies directed against EGFR that can potentially be beneficial for the treatment of OED[22]. The present study determines and characterizes EGFR expression in low and high grade epithelial dysplasia, comparing it with its expression in normal mucosa and in OSCC of different stages.

Importantly, the present study observed that OEDs tend to over express EGFR, regardless of its degree, in the same way as OSCC.

Table 1: Summary of patients' characteristics

Samples	Gender X		$\overline{X}$	Age	
	M n (%)	F n (%)		±SD	Min., Max.
NM (n=9)	5 (56)	4 (44)	19	± 1922222	15, 21
LGD (n=35)	16 (46)	19 (54)	49	± 16.70027	9, 74
HGD (n=19)	10 (53)	9 (47)	56	± 14.21617	17, 79
OED (n=54)	26 (48)	28 (52)	52	± 16.05964	9, 79
OSCC S1 (n=24)	18 (75)	6 (25)	61	± 10.76596	38, 82
OSCC S2 (n=16)	11 (69)	5 (31)	53	± 8.916044	40, 73
OSCC S3 (n=6)	4 (67)	2 (33)	60	± 9.537645	50, 70
OSCC S4 (n=14)	8 (57)	6 (43)	49	± 8.218179	36, 67
OSCC (n=60)	41 (68)	19 (32)	56	± 10.74323	36, 82

NM., Normal Mucosa; LGD., Low grade Dysplasia; HGD., High grade Dysplasia; OED., Oral Epithelial Dysplasia; OSCC., Oral Squamous Cell Carcinoma; S1., Stage 1; S2., Stage 2; S3., Stage3; S4., Stage 4.

Table 2: Comparison of EGFR expression between OED groups, OSCC groups and NM.

Samples	LE n (%)	HE n (%)	p-value	Total n (%)
NM	9 (100)	0 (0)	0.217044	9 (100)
LGD	12 (34)	23 (66)		35 (100)
HGD	5 (26)	14 (74)		19 (100)
OED	**17 (31)**	**37 (69)**		**54 (100)**
OSCC S1	9 (37,5)	15 (62,5)	< 0.00001	24 (100)
OSCC S2	4 (25)	12 (75)		16 (100)
OSCC S3	0 (0)	6 (100)		6 (100)
OSCC S4	3 (21)	11 (79)		14 (100)
OSCC	**16 (27)**	**44 (73)**		**60 (100)**

LE., Low EGFR expression; HE., High EGFR expression; NM., Normal Mucosa; LGD., Low grade Dysplasia; HGD., High grade Dysplasia; OED., Oral Epithelial Dysplasia; OSCC., Oral Squamous Cell Carcinoma; S1., Stage 1; S2., Stage 2; S3., Stage3; S4., Stage 4.

Some previous studies that evaluated OPMD samples with or without dysplasia, without considering the degree, reported positivity for EGFR in 62.5% of leucoplakia[25] or high expression of this marker in 71% of OPMD samples[18]. In addition, EGFR expression has been reported to increase with the progression of dysplasia lesions to OSCC[24], and EGFR overexpression was found to occur exclusively in leucoplakia that transformed into OSCC[26]. In relation to the degree of dysplasia, a lower expression of EGFR was reported in cases with low-grade OED that did not transform to OSCC compared to the group in which there was malignant transformation[26].

It would be expected to observe positive expression of EGFR in the oral mucosa, as there are studies that indicate that it is involved in the repair of this mucosa[25,27,28]. In contrast to these data, in the present study all normal tongue mucosa samples were negative for EGFR. In relation to OSCC, the result was consistent with the literature that reports high expression in 60-98% of cases[17]. These observations were important to compare with the OED samples, as it is expected that in these cases, as well as in OSCC, an imbalance occur mediated by different genetic alterations that cause an increase in EGFR and/or an increase in the activity of these receivers[15].

As the evaluation of OED grade remains the standard strategy to predict the potential of malignant transformation, the analysis of EGFR expression in OPMD samples with low and high grade OED, contrasted with OSCC and NM, and contributes to consolidating the studies reported in the literature. Initially, Benchekroun et al. 2010[18] suggested that increased EGFR copy number was a risk factor for malignant transformation. However, later Nankivellet al.[22], in their follow-up study of patients in relation to the transformation to OSCC, identified that high EGFR expression was also a risk factor in patients with OED of different degrees, which suggests that these

Table 3: Expression of EGFR according to location in epithelial layers

	LGD (n=35)		HGD (n=19)		Total ODE (n=54)	
	Neg.-Weak	Mod.-int.	Neg.-Weak	Mod.-int.	Neg.-Weak	Mod.-int.
Epithelial Layers	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Basal	6 (17)	29 (82)	5 (26)	14 (74)	11 (20)	43 (80)
Parabasal	8 (23)	27 (77)	5 (26)	14 (74)	13 (24)	41 (76)
S. Spinosum	25 (71)	10 (29)	11 (58)	8 (42)	36 (67)	18 (33)
S. Superficial	35 (100)	0 (0)	19 (100)	0 (0)	54 (100)	0 (0)

S., Stratum; LGD., Low grade Dysplasia; HGD., High grade Dysplasia; OED., Oral Epithelial Dysplasia; Neg.-Weak., Negative-Weak staining; Mod.-Int., Moderate-Intense staining

Table 4: Association between EGFR expression and clinic pathologic parameters of OED and OSCC samples.

	LGD (n=35)			HGD (n=19)			OED (n=54)			OSCC (n=60)		
	LE	HE	p-value	LE	HE	p-value	LE	HE	p-value	LE	HE	p-value
Gender												
F	3	16	0.817	2	7	0.701	5	23	0.634	5	14	0.813
M	3	13		3	7		6	20		12	29	
Age (Years)												
< 50	3	13	0.817	1	3	0.946	4	16	0.959	5	8	0.360
> 50	3	16		4	11		7	27		12	35	
Site												
Lower lip	3	3	0.338	0	0	0.449	3	3	0.189	ND	ND	
Cheek	1	8		0	3		1	11		ND	ND	
Gingiva	1	7		1	4		2	11		ND	ND	
Tongue	1	9		1	4		2	13		ND	ND	
Palate	0	1		0	1		0	2		ND	ND	
Mouth floor	0	1		2	1		2	2		ND	ND	
Superior lip	0	0		1	1		1	1		ND	ND	

LGD., Low grade Dysplasia; HGD., High grade Dysplasia; OED., Oral Epithelial Dysplasia; OSCC., Oral Squamous Cell Carcinoma; LE., Low EGFR expression; HE., High EGFR expression; ND., No data.

high-risk lesions should be treated with EGFR antagonists. Meanwhile, other studies suggest that it would be interesting to evaluate the interaction between EGFR and COX2, CD9 and CD151[29-31], which could lead to multimodal approaches for chemoprevention in the treatment of OPMDs[22].

In the present study, it was observed that moderate to intense EGFR labeling in positive OED samples was preferentially located in the basal and suprabasal strata, although this information has not yet been reported in the literature. Regarding the clinical characteristics, no association was observed between high or low EGFR expression in relation to age, gender or location of HGD and LGD. On the other hand, studies that evaluated the association between positivity and EGFR found a high risk in relation to lesions located on the tongue and floor of the mouth[25].

Loss of heterozygosity (LOH) in 3p and/or 9p may be present in OPMD without dysplasia or with low-grade dysplasia, which is used as an independent marker of OED to characterize patients with high risk of malignant transformation[32]. Considering high-risk patients such as those with LOH, a study determined that EGFR blockade with Cetuximab could give a significant histological and clinical resolution in some of these patients with moderate to severe dysplasia lesions[33].

Conclusion

There is a tendency for both LGD, HGD and OSCC to have a high EGFR expression, but not normal oral mucosa. This confirms that EGFR overexpression is an intrinsic factor of dysplastic and neoplastic tissues, which serves as an early biological marker of these pathologies[34]. Based on these data, it is suggested that more follow-up studies be conducted to identify EGFR overexpression as a risk factor for malignant transformation, with a greater number of LGD and HGD samples and the experimental use of EGFR blockers for the treatment of OPMDs with OED that overexpress this marker.

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