



REVIEW

Carolina Somarriva.¹
Alejandra Fernández.^{1,2}
Jorge Candia.¹
Javier Campos.²
Daniela Albers.^{2,3}
Jorge Briceño.¹

1. Facultad de Odontología. Universidad Andres Bello. Chile.
2. Centro de Investigación Biomédica. Universidad de Los Andes, Chile.
3. Facultad de Odontología. Universidad Mayor, Chile.

Corresponding author: Alejandra Fernández. Facultad de Odontología. Universidad Andres Bello. Avda Echaurren 237. Santiago, Chile. Phone: (+56-9) 98796026. E-mail: fernandez.alejandra@gmail.com

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Dysregulation and detection methods of EGFR in oral cancer. A narrative review.

Abstract: Epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein, with an intracellular domain and tyrosine kinase function (TK) involved in cell proliferation. Dysfunctions in EGFR signaling pathways have been associated with oral malignant tumors such as oral squamous cell carcinoma (OSCC). Dysfunctions of EGFR may result from: increased EGF ligand; EGFR overexpression and copy number gain of the *EGFR* gene (*EGFR* CNG); *EGFR* mutations; failure in the downregulation of EGFR; and EGFR crosstalk. Of these alterations, overexpression of EGFR is by far the most studied dysfunction in OSCC. Clinicians should identify possible alterations of EGFR in the oral mucosa of patients, as EGFR can act as a biomarker for the diagnosis and prognosis of OSCC. Currently, there are several methods and techniques for detecting EGFR. Immunohistochemistry (IHC), fluorescence in situ hybridization (FISH) and polymerase chain reaction (PCR), are used to identify overexpression of EGFR, *EGFR* CNG and EGFR mutations, respectively. Detection of EGFR as a biomarker is key to identify any oral malignant transformation. Consequently, it becomes imperative to implement a non-invasive and inexpensive method of early diagnosis for OSCC in clinical practice.

Keywords: *Epidermal Growth Factor Receptor, Mouth Neoplasm, Mutation.*

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INTRODUCTION.

In 1962, Dr. Cohen studied a protein extracted from the submandibular glands responsible for the early growth of the incisors and the eyelid in mice. In 1979, Cohen and Carpenter named this protein epidermal growth factor (EGF).^{1,2} EGF binds to its EGFR receptor by means of a covalent bond type.¹ EGFR is a protein encoded by a gene located on the short arm of chromosome 7 (182-184), region p14-p12.² Its protein is a transmembrane glycoprotein receptor with 1186 amino acids and 170 kDa. Through the TK cytoplasmic domain, they transduce signals from the cell membrane to the nucleus, controlling cell proliferation, differentiation, survival and motility.^{1,3,4}

EGFR has been associated with tumorigenic, proliferative, apoptotic, invasive and metastatic processes of

epithelial origin.⁵ EGFR is involved in the pathogenesis of non-small-cell lung carcinoma (NSCLC), colorectal carcinomas, and oral squamous cell carcinoma (OSCC).⁶⁻¹¹ The use of EGFR as a molecular biomarker in conjunction with molecules involved in signal transduction are ideal targets for OSCC therapies and useful for early diagnosis, prognosis and treatment of cancer.^{12,13}

In the last decade, the association of EGFR with carcinomas has increased the interest in its genomic evaluation. Molecular detection is performed through epithelial cell membranes using diverse techniques depending on the objectives of each study.

The aim of this review is to describe the alterations in EGFR and identify the methods most commonly used to detect it in oral cancer.

DYSREGULATION OF EGFR IN ORAL CANCER.

Dysregulation of EGFR in cancer has been extensively studied.^{6,9,14} Mechanisms involved in the dysregulation of EGFR are many, including:

- 1) Increased EGF ligand;¹⁵
- 2) Overexpression of EGFR and *EGFR* CNG;^{9,16}
- 3) *EGFR* mutations;¹⁷
- 4) Failure in the downregulation of EGFR;¹⁸
- 5) EGFR crosstalk.^{7,19}

Increased EGF ligand

Activation or inhibition of EGFR is determined by the binding of its ligands. Molecules that bind to EGFR include EGF, amphiregulin, epigen, transforming growth factor alpha (TGF- α), betacellulin, heparin-binding EGF, epiregulin, neuregulin or heregulin and insulin-type growth factor. However, the main ligand of EGFR is EGF.^{15,20} EGF is a single polypeptide chain comprising 53 amino acids.¹ Increased synthesis of EGF has been associated with a number of tumors, including head and neck cancer (HNC).^{21,22} EGF contributes to the growth of malignant tumors by stimulating cell proliferation and migration. It also participates in the dysregulation of autophagic activity and tumor metastasis through metalloproteinases.¹⁸

Some therapies with monoclonal antibodies have had an anti-proliferative effect on cancer cells expressing EGFR.^{23,24} Cetuximab (ErbiximTM), a chimeric monoclonal antibody, recognizes an epitope in the extracellular domain III of EGFR. It has been widely used in clinical studies due to its ability to inhibit EGFR by occupying the ligand-binding site, reducing the proliferation of cancer cells. However, it has been shown to have better anti-tumor effects when combined with chemotherapy or radiotherapy.²⁵

Overexpression and increase in copy number of the EGFR gene

Hyperactivation and dimerization of EGFR play an important role in the regulation of the PI3K/AKT/mTOR pathway. The activation of this pathway revert autophagy through inhibition of Beclin-1 tyrosine phosphoryla-

tion, which in turn prevents the formation of class III PIK3 complex, promoting epithelial neoplasm progression. Hyperactivation of the PI3K/AKT/mTOR pathway is associated with the development, growth and proliferation of up to 50% of cancers.²⁵ EGFR overexpression has been observed in epithelial-origin tumors as well as in NSCLC, renal, ovarian, breast, prostate cancer and colorectal carcinomas.

In HNC, 70-90% of neoplasms present overexpression of EGFR.⁹ In OSCC, there has been observed an increase in EGFR expression in the cell plasma membrane of oral keratinocytes,²⁶ resulting in a poor prognosis, high recurrence and lower survival rate.^{9,16,26}

EGFR mutations

EGFR mutations can be classified according to the specific region of the receptor they affect: extracellular, intracellular or TK domain. Deletion type mutations affecting the sequence encoding the N-terminal region, deletions of exons 2-7, 12-13, 14-15, 25-27, 25-28; duplications of exons 2-7, 18-21, and point mutations have been described. As a result, small deletions and insertions alter codon sequence producing proteins with aberrant function. These aberrant proteins may have a decreased activity or maintain a constitutive activation.^{3,17,27-30}

The mutated variant, known as *EGFR* variant III (*EGFRvIII*), encoded by *EGFRvIII*, has a deletion of exons 2-7, which encode the extracellular domain of ligand binding. The altered protein is constitutively active with slow degradation, allowing more time to interact with its ligand. It is the most common *EGFR* mutation and the best described in relation to various malignancies. *EGFRvIII* has been associated with increased tumor cell proliferation in mouse model and it has been observed that its presence is associated with a lower response to treatment with radiation therapy.^{3,16,30,31} McItyre *et al.*³ studied the expression of *EGFRvIII* in OSCC, noting that this is overexpressed in 2% of patients. Melchers *et al.*³¹ analyzed 531 cases of HNC and found no difference in the prevalence of the mutation compared to healthy controls. Khattri *et al.*²⁸ found that only 2 (0.31%) out of 638 cases

had the *EGFRvIII* mutation. Therefore, this type of mutation is extremely rare in HNC and OSCC.^{3,28,31}

Mutations in exons 18, 19, 20 and 21 correspond to rare amino acid variations of the TK intracellular domain. About 90% of *EGFR* mutations are deletions located in exon 19 and the L858R point mutation of exon 21.²⁹ Mutations in exon 20 encode proteins that normally are located after the C-helix of TK domain. This occurs in 4% of all *EGFR* mutations, with T790M substitution being the most prevalent, representing 50% of all mutations in exon 20.³²

Hsieh *et al.*³³ studied *EGFR* in patients with OSCC who chewed betel nut, finding that 30.36% had silent mutation at nucleotide 2607 in exon 20. This mutation does not alter the amino acid sequence and results in a mutation at codon 787 (Q787Q). They also identified two types of silent mutations in exon 21 which corresponded to 1.79% of the cases; however, they found no mutations in exons 18 and 19. Furthermore, Nagalakshmi *et al.*¹⁷ studied *EGFR* mutations in OSCC, finding that the samples studied showed mutations in exons 18 (nucleotides G2155C, G2176A), 19 (nucleotide C2188G) and 21 (nucleotide G2471A), with frequencies of 44.96%, 32.55% and 65.11%, respectively.

Defects in EGFR downregulation

In normal conditions, after ligand binding, cytoplasmic tyrosine residues from EGFR are autophosphorylated, producing binding zones for various proteins. The recruitment of these proteins occurs in catalytic domains and/or scaffolds actively involved in cell signaling. An important pathway for deactivating TK receptors is downregulation. In this process, the activated receptor is internalized by the plasma membrane by means of endocytosis. Then, it is ubiquitinated and transported to the lysosomes where it is degraded by acid hydrolases.

When the TK domain is not deactivated appropriately, a failure in normal activity and operation of the receptor can occur.^{34,35} Yang *et al.*³⁶ suggested that the ability of mutated EGFR to escape downregulation may be due to lack of: ubiquitin binding, dysregulation of kinase associated with cyclin-G, and reduced levels of

CD82 (metastasis suppressor).

Zhen *et al.*³⁴ studied the effect of curcumin on cultivated cells from patients diagnosed with OSCC. The study concluded that curcumin revert growth of tumor cells by inhibiting EGFR phosphorylation. Curcumin is known for inhibiting the growth, invasion and metastasis of malignant cells and for inducing apoptosis in breast cancer.

Another molecule that has been studied in conjunction with EGFR, is E-cadherin. This molecule is responsible for preserving integrity and cell morphology. Wang *et al.*³⁷ observed that *in vitro* reduction of E-cadherin increases the upregulation of EGFR transcription. This suggests that loss of E-cadherin can induce proliferation of HNC by activating EGFR and its signaling pathways to the nucleus. It is essential to determine if the increase in E-cadherin plays a role in the downregulation of EGFR.

EGFR crosstalk

Cytoplasmic and nuclear signaling pathways can be activated by proteins acting at similar levels and conditions. This feature is known as crosstalk and is a form of evolutionary compensation to avoid a receptor being activated by a single ligand. Crosstalk between EGFR and other members of the ErbB family, cytokine receptors, ion channels, G protein-coupled receptors and various cell adhesion molecules has been described in the literature.^{38,39} The integrin family phosphorylates the TK domain increasing the receptor activity.⁴⁰ Zein *et al.*³⁸ studied the relationship that existed between the EGF-EGFR complexes and nerve growth factor with its receptor, finding that there is a bidirectional crosstalk between ligands and their receptors.

In HNC it has been observed that some of the ligands that bind to G protein-coupled receptors activate EGFR pathway, contributing to carcinogenesis.³⁹ It is suggested that stimulation of gastrin-releasing peptide receptor activates EGFR and modulates the growth and invasion of HNC.⁴¹ Egloff *et al.*¹⁹ characterized the expression and signaling of estrogen receptors (Era and Erb) in HNC in relation to the EGF-EGFR complex. At the level of signal transduction and transcription, they found that Era and Erb receptors were expressed and stimulated in HNC.

Table 1. Laboratory technique by author according to EGFR alteration.

Type of Alteration	Laboratory Technique	Authors
Increased EGF ligand	ELISA	Zhang <i>et al.</i> ⁴¹ 2014
	IHC	Naik <i>et al.</i> ⁴⁸ 2011
Overexpression of EGFR	IHC	Aquino <i>et al.</i> ⁴⁴ 2012
	WB	Zhang <i>et al.</i> ⁴¹ 2014
Copy Number Gain of EGFR gene	RT-PCR	Wang <i>et al.</i> ³⁷ 2011
	FISH	Huang <i>et al.</i> ⁴⁵ 2012 Aquino <i>et al.</i> ⁴⁴ 2012 Szabó <i>et al.</i> ⁹ 2011
	CISH	Bernardes <i>et al.</i> ⁴⁷
	Real Time-PCR	Bagan <i>et al.</i> ²⁶ 2012
EGFR mutations	PCR	McIntyre <i>et al.</i> ³ 2012 Nagalakshmi <i>et al.</i> ¹⁷ 2014 Szabó <i>et al.</i> ⁹ 2011
	IHC	Khattri <i>et al.</i> ²⁸ 2014 Szabó <i>et al.</i> ⁹ 2011
	HRM	Do <i>et al.</i> ²⁷ 2008
	Q-PCR	Khattri <i>et al.</i> ²⁸ 2014
	RT-PCR	Khattri <i>et al.</i> ²⁸ 2014 Melchers <i>et al.</i> ³¹ 2014
	Defects in EGFR downregulation	MMF
EGFR crosstalk	WB	Zhen <i>et al.</i> ³⁴ 2014
	WB	Egloff <i>et al.</i> ¹⁹ 2009 Thomas <i>et al.</i> ³⁹ 2006
	ELISA	Zein <i>et al.</i> ³⁸ 2010

ELISA: Enzyme-linked immunosorbent assay. **IHC:** Immunohistochemistry. **WB:** Western Blot. **RT-PCR:** Polymerase chain reaction with reverse transcriptase. **FISH:** fluorescent in situ hybridization. **CISH:** Chromogenic In Situ Hybridization. **Real time-PCR:** Real-time polymerase chain reaction. **PCR:** Polymerase chain reaction. **HRM:** High resolution melting. **Q-PCR:** Quantitative polymerase chain reaction. **MPM:** Multi-site Phosphorylation Model

DETECTION METHODS OF EGFR IN ORAL CANCER.

Clinical significance of EGFR detection in oral mucosa lies in its role as a biomarker or indicator of malignant transformation, diagnosis, progression and prognosis of OSCC. The National Cancer Institute defines a biomarker as any molecule found in fluids or tissues that is a sign of a physiological or pathological process.⁴²

Identification of EGFR as an indicator of malignant transformation is based on its overexpression in potentially malignant samples as leukoplakia and oral epithelial dysplasia (OED).¹⁶ The expression of EGFR varies according to the degree of OED;

expression is greater with increasing malignancy. Consequently, EGFR can be considered as a marker of cell epithelial proliferation, of OED, and as the onset of progression from dysplasia to OSCC.⁴³ Bagan *et al.*²⁶ reported that *EGFR* CNG is a potential marker for predicting malignant transformation of OED. The authors noted that *EGFR* CNG was significantly higher in malignant lesions and in non-homogeneous leukoplakia compared to homogeneous leukoplakia. Regarding OSCC, Aquino *et al.*⁴⁴ evaluated overexpression of EGFR protein and *EGFR* CNG by immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH), respectively. They found high expression of EGFR and *EGFR* CNG (14%), confirming the important role

of EGFR as a biomarker in OSCC.

Huang *et al.*⁴⁵ found that *EGFR* CNG in samples of OSCC were associated with characteristics of local invasion, including bone and perineural invasion. In oral cancer, overexpression of EGFR has been associated with chemoresistance and a poor prognosis.¹⁸ *EGFR* CNG is a late event in oral carcinogenesis. In OSCC it ranges from 9% to 56% of cases, being more frequent in T3 and T4 stages.⁴⁶

However, other authors have concluded that there is no association between EGFR expression and *EGFR* CNG in OSCC. Moreover, neither of these changes was associated with clinicopathological features of OSCC. EGFR may be useful as a predictive marker, but it seems crucial to determine the best method to assess its relationship with alterations and cancer.⁴⁷ There are several molecular techniques with variable specificities and sensitivities for detecting EGFR in oral mucosa and its association with various events in OSCC, (Table I).^{3,9,17,19,20,27,28,31,34,35,38,39,41,45,47,48}

The most widely used method for EGFR detection is IHC. It is a simple and relatively quick technique. However, it is semi-quantitative in nature, and can be affected by operator bias. Polymerase chain reaction (PCR) has been used to detect EGFR mutations in normal and neoplastic tissues.^{3,17,31,35,49} In oral cancer specific tests to evaluate deletions, silent mutations or changes at amino acid level encoded in exons 18 to 21 have been used.³³ Bagan *et al.*²⁶ studied the genomic amplification of EGFR in frozen tissue samples. Using Real-Time-PCR, they found a high rate of *EGFR* CNG in oral cancer when compared with potentially malignant lesions. It is also possible to study *EGFR* CNG by FISH and analyze somatic mutations in exons

18 to 21 by high resolution melting (HRM). The latter method is fast, easy to use and inexpensive, but it is rarely used.²⁷ Even with these advantages, more research with larger samples and new types of analyses is needed due to the high rate of false negatives.⁵⁰

A relatively new diagnosis method is the identification of EGFR biomarkers using bio-nano-chip (BNC). The BNC sensor integrates multiple laboratory processes in a three-step microfluidic platform. A simultaneous analysis of the surface expression of the biomarker and cell morphology is performed using intensity and multiple key parameters. First, oral cells extracted by cytology are placed in the sensor by pressure driven flow. Captured cells are stained with fluorescent and immunoreactive dyes. Finally, stained cells are subjected to fluorescence analysis by 3D microscopy of the membrane surface. Researchers have shown that the BNC sensor correctly identifies premalignant and malignant lesions in less than 45 minutes. However, authors emphatically suggest creating a wider range of biomarkers for early detection of cancer and dysplasia.⁵¹ The Western Blot technique (WB), immunoblot or immunoblotting, was used to assess EGFR dysregulation.³⁶ In the same way, Wang *et al.*³⁷ and Egloff *et al.*¹⁹ besides studying EGFR, used WB to quantify overexpression of E-cadherin and of the estrogen receptor, respectively.

CONCLUSION.

The most studied EGFR dysregulations in OSCC are receptor overexpression and mutations by means of IHC, FISH and PCR. These techniques are costly and complex, so it is crucial to develop a low-cost, non-invasive and easy to use method.

Desregulación y métodos de detección del EGFR en cáncer oral. Revisión narrativa.

Resumen: El receptor del factor de crecimiento epidérmico (EGFR) es una glicoproteína transmembrana, con un dominio intracelular y función tirosina quinasa (TK) que participa en la proliferación celular. Las fallas en las vías de señalización del EGFR se han asociado con la formación de tumores malignos orales como el carcinoma oral de células escamosas (COCE). El incorrecto funcio-

namiento del EGFR puede producirse por: aumento del ligando EGF; sobreexpresión del EGFR y ganancia en el número de copias del gen EGFR (GNC *EGFR*); mutaciones del EGFR; falla en la regulación negativa del EGFR; y diafonía del EGFR. De las alteraciones mencionadas, la sobreexpresión de EGFR es por lejos la disfunción más estudiada en COCE. Para el clínico es importante poder identificar las posibles alteraciones del EGFR en la mucosa oral del paciente, esto debido a que el EGFR puede

actuar como un biomarcador de diagnóstico y pronóstico para COCE. En la actualidad existen diversos métodos para detectar el EGFR. La inmunohistoquímica (IHC), la hibridación fluorescente in situ (FISH) y la reacción en cadena de la polimerasa (PCR), son técnicas utilizadas para identificar la sobreexpresión del EGFR, GNC EGFR y mutaciones del EGFR, respectivamente. La necesidad de

detección de estas alteraciones se debe a la trascendencia del EGFR como biomarcador de transformación maligna. Lo anterior, hace necesario implementar un método de diagnóstico precoz para COCE que sea no invasivo y de bajo costo para la práctica clínica.

Palabras clave: *Receptor del Factor de Crecimiento Epidérmico, Neoplasias de la Boca, Mutación.*

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