BMJ open
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EGFR status in oral squamous cell carcinoma: comparing immunohistochemistry, FISH and CISH detection in a case series study

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ABSTRACT

Objectives: To compare the immunohistochemistry (IHC) expression of epidermal growth factor receptor (EGFR) in oral squamous cell carcinomas (OSCC) with the gene amplification evaluated by fluorescence in situ hybridization (FISH) and chromogenic in situ hybridization (CISH) and their association with clinicopathological parameters. Additionally, we tested the sensibility and specificity of CISH in comparison with FISH.

Design: Case series study

Setting: Oral surgery and pathology department in a school of dentistry.

Participants: 52 patients with histopathological diagnosis of OSCC.

Methods: Tumour tissue samples from 52 patients with OSCC were evaluated by IHC, FISH and CISH using tissue microarray technology. Clinicopathological data from all patients were collected.

Results: EGFR+ rates were 53.8% (28/52) by IHC, 5.8% (3/52) by CISH and 15.4% (8/52) by FISH. Amplification detected by CISH and FISH with IHC negative occurred in 3.8% (2/52), and one case (1.9%) showed amplification detected by CISH and FISH and protein overexpression concomitantly. There were 9.6% FISH+ cases with IHC and CISH negative rates and 6/8 (75%) FISH+ and also EGFR+ cases; however, an association between protein expression and gene amplification was not found for both techniques. IHC and FISH rates were not associated with clinicopathological features. CISH+ rates were associated with T3–T4 status. Compared with FISH assay, CISH reached a sensitivity of 37.5% and specificity of 100%.

Conclusions: There is no association between EGFR expression and gene amplification in OSCC when the IHC is driven to external epitopes of the protein. Although CISH demonstrates specificity, technical problems may influence sensibility when compared with FISH.

ARTICLE SUMMARY

Article focus

- This study compares immunohistochemistry, chromogenic in situ hybridization (CISH) and fluorescence in situ hybridization (FISH) in the evaluation of epidermal growth factor receptor (EGFR) status in oral squamous cell carcinoma (OSCC). It shows that there is no association among these methods and discusses the limitations of each of them.
- The authors suggest that other mechanisms that alter the protein expression, which are not related to amplification, need to be investigated in OSCC.

Key messages

- There is no association between EGFR expression and gene amplification in OSCC when the immunohistochemistry is driven to external epitopes of the protein.
- Although CISH demonstrates specificity in these samples, technical problems may influence sensibility when compared with FISH.

Strengths and limitations of this study

- The strength of this study is the comparison among three standardised methods of EGFR investigation. This signalling is becoming attractive in the therapy of head-and-neck squamous cell carcinoma.
- The following limitation should be considered: preanalytical factors might have influenced the signal intensity of CISH.

INTRODUCTION

Epidermal growth factor receptor (EGFR) signalling participates in the regulation of cell proliferation and differentiation during development and, in tumour cells, contributes to proliferation, invasion and metastasis formation.[1 2] It is frequently expressed in many types of cancer including the head-and-neck squamous cell carcinoma (HNSCC). As its overexpression is frequently associated with poor clinical outcome, the receptor is becoming attractive in the therapy of this neoplasm.[3 4]

Comparative studies of EGFR detection by immunohistochemistry (IHC) and fluorescent in situ hybridization (FISH) have shown a high concordance rate. Chromogenic in situ hybridization (CISH) was introduced a few years ago as an alternative to FISH in view of some advantages, such as evaluation in the conventional bright-field microscopy system, permanent record and preserved morphological features.[5][6] However, to date, no studies have been published regarding EGFR copy numbers detected by FISH concomitant to CISH and the evaluation of this protein expression in oral squamous cell carcinoma (OSCC).

The objective of this study was to investigate the profile of expression of EGFR protein and correlate this finding with the gene status evaluated by two methods of hybridization (CISH and FISH) in tumour samples of OSCC. Clinicopathological parameters were also included in the evaluation of the tumours. Additionally, the accuracy of CISH has been compared with FISH.

MATERIALS AND METHODS
Patients' eligibility
Patients with a histopathological diagnosis of OSCC were enrolled in this study. Clinical data, such as age, gender, symptoms, location and extension of the tumour, nodal involvement and tobacco and alcohol habits, were obtained from medical records. The tumour samples were obtained from incisional biopsy before any adjuvant therapy.

This study was approved by the local Research Ethics Committee, and a signed, informed consent was obtained from all participants.

Immunohistochemistry (IHC)
IHC for the detection of the EGFR antigen was performed using the monoclonal antibody clone 31G7 (Zymed Laboratories Inc, San Francisco, California, USA) and sections of oral mucosa were used as positive control. Briefly, 4 µm sections were plated on histological slides treated with 3-aminopropyltriethoxy-silano, followed by the deparaffination, hydration and blocking of intrinsic enzymatic activity. Slides were then immersed in pepsin 10% at 37°C. After washing in distilled water, sections were subsequently incubated in the primary antibody (1:100 dilution) at room temperature for 60 min. After rinsing in Tri-HCl buffer, sections were incubated for 30 min at room temperature with biotinylated multilink swine antigoat, mouse and rabbit immunoglobulin (LSAB Kit, DaKo, Carpinteria, California, USA). The reactions were revealed by applying 3,3'-diaminobenzidine (DAB) in chromogen solution (Dako; Carpinteria, California, USA). The sections were counterstained with Mayer's haematoxylin and mounted in Permount (Fisher Scientific; New Jersey, USA). Semiquantitative assessment of the immunohistochemical stain results were performed by a pathologist who was unaware of the clinicopathological details and the gene amplification status. EGFR expression was evaluated according to a previously defined four-point scale based on the immunolabelling of tumour cell membranes proposed by Diniz-Freitas et al[7] as follows: 0 (no labelling or labelling in <10% of tumour cells); (1) (weak labelling, homogeneous or patchy in >10% of the tumour cells); (2) (moderate labelling, homogeneous or patchy in >10% of the tumour cells); (3) (intense labelling, homogeneous or patchy in >10% of the tumour cells). These scores were subsequently grouped into two categories: negative (0 or 1) and positive labelling (2 or 3).

Tissue microarray (TMA)
Representative core tissue sections of strong immunoexpression of EGFR were taken from paraffin blocks from each patient and arranged in a new tissue microarray (TMA) block using the Manual Tissue Arrayer (Beecher Instruments, Silver Spring, Maryland, USA). As previously suggested by Monteiro et al[8], we selected the area of interest avoiding necrosis and keratin pools. In the negative cases, a representative area of the tumour was selected considering the histological graduation. One core with 1 mm diameter was used from each sample. In an attempt to perform both FISH and CISH assays, 4 µm thick sections were obtained.

Fluorescent in situ hybridization (FISH)
A Zytolight Spec EGFR/CEN 7 Dual Color Probe (Zytovision, Bremerhaven, Germany) was used to perform FISH. After deparaffinisation in two rinses of 100% xylene for 5 min each, the slide was rehydrated through two rinses of 100% alcohol for 5 min each. Next, the slide was treated with 0.2 mmol/l HCl for 20 min, distilled water for 2 min and 2× standard saline citrate for 3 min. The slide was submitted to the pretreatment buffer (Saline-sodium citrate (SSC) buffer: 0.3 mmol/l sodium chloride and 0.03 mmol/l sodium citrate) for 30 min at 82°C in a water bath and then for 36 min at 37°C in the protease digestion in protease buffer (0.05 mmol/l Tris-HCl at pH7.8, 0.01 mmol/l ethylene diamine tetra-acetic acid and 0.01 mmol/l NaCl). Finally, the slide was placed in 10% formalin in phosphate-buffered saline (PBS) for 10 min. Dehydration was performed in 70, 85 and 100% ethanol, consecutively. Both FISH probes and target DNA were denatured simultaneously for 5 min at 75°C and incubated overnight at 37°C. For signal detection, the slide was placed in the posthybridization wash buffer (SSC and NP40) for 3 min at 74°C and counterstained in 2× SSC/0.03 µg/ml 4,6-diaminido-2-phenylindole dihydrochloride for the identification of the nucleus.

Chromogenic in situ hybridization (CISH)
CISH was performed on 4 µm-thick formalin-fixed, paraffin-embedded tumour samples. Briefly, tissues were deparaffinised in xylene and immersed first in ethanol and later in CISH Tissue Heat Pretreatment Solution (Invitrogen Corporation, Camarillo, California, USA) at

98°C for 21 min. The slides were immediately washed with distilled water for 2 min. Enzymatic digestion was performed by incubating sections within pepsin for 10 min at room temperature. The slides were then washed, dehydrated with graded ethanol and air-dried. A ready-to-use Spot-Light EGFR probe (Invitrogen) 15 µl was applied to the coverslips, which were placed on the sections, and the edges were sealed. The slides were denatured on a hot plate (94°C) for 5 min and hybridization was performed overnight at 37°C in a humidified chamber (Dako Hybridizer:Dako, Carpinteria, California, USA). A stringent wash was performed with 0.5× standard saline citrate at 75°C for 5 min; a PBS/Tween 20 wash was performed twice for 2 min. Sections were blocked with 3% H_2O_2, diluted with methanol for 10 min and PBS wash was performed twice for 2 min. The unspecific staining was blocked by applying the Cas-Block (Spot-Light CISH detection Kit) and by incubating for 10 min. After incubation with a Mouse anti-Dig antibody for 30 min at room temperature, the procedure was continued by incubation with a polymerised HRP-antimouse antibody and substrate-chromogen solution (DAB) for 30 min at each step and counterstained with haematoxylin for 5 s. The tissues were dehydrated in ethanol and coverslipped in Tissue-Tek Prisma/Film (Sakura Finetek Inc, Torrance, California, USA). The Spot-Light chromosome 7 centromeric probe has been used to check for polysomy (Invitrogen).

Evaluation of CISH and FISH results

Gene status was determined according to the manufacturer's criteria and classified in three categories: no amplification (1–5 copies of the gene present per nucleus in >50% of cancer cells); low amplification (6–10 copies of the gene, or a small gene cluster, present per nucleus in >50% cancer cells); amplification (>10 copies, or large clusters, of the gene present per nucleus in >50% cancer cells). The scores were subsequently grouped into two categories: no amplification and amplification. The whole extension of the core has been evaluated counting a mean of 1 nuclei per sample.

Statistical analysis

Categorical variables were analysed by χ^2 test or Fisher's exact test when appropriate. The sensitivity and specificity of CISH were calculated using FISH as the gold standard. The level of significance was set to 5% for all tests. Statistical analysis was performed by SPSS software, V.17.0 (SPSS, Chicago, Illinois, USA).

RESULTS
Clinicopathological results

Clinicopathological and molecular information is described in table 1. The sample comprised 52 cases of OSCC (40 male, 12 female) with mean ages of 56.3 years (range from 16 to 80). The T-staging and N-staging of the tumours were described according to the AJCC (American Joint Committee on Cancer)/UICC (Union for International Cancer Control) classification for oral cavity carcinomas.[9] Malignancy grade was as follows: 17 (32.7%) well-differentiated, 14 (26.9%) moderately differentiated and 21 (40.4%) poorly differentiated tumours. The most common affected sites were the tongue and/or floor of the mouth (75%); the majority of these lesions presented nodal involvement and were classified as poorly differentiated. Smokers comprised 81% of the patients, of which 18.8% revealed the consumption of more than 20 cigarettes/day. Alcohol habit was reported by 64.3% of the patients. A tendency of the smokers to present high-grade tumours was observed, chiefly when the consumption was above 20 cigarettes/day (p<0.05). The mean of follow-up was 6 months.

Immunohistochemistry, FISH and CISH results

The pattern of the immunoexpression was a distinctive brown staining in the cytoplasmic membrane of the neoplastic cells, and 28 (53.8%) cases were positive (figure 1A). CISH detected gene amplification in 3 (5.8%) cases and FISH in 8 cases (15.4%) (figure 1B,C). Amplification detected by CISH and FISH without protein overexpression occurred in 2 (3.8%) cases. One (1.9%) case showed amplification detected by CISH and FISH and protein overexpression concomitantly. Five (9.6%) cases revealed FISH amplification and protein overexpression without CISH amplification.

EGFR expression and amplification (CISH and FISH) were more frequent in low-grade tumours, but without significance (p>0.05). Neither EGFR expression nor FISH results showed association with clinicopathological features. Amplification detected by CISH was associated with T3-T4 status (p=0.02). There was no association between protein expression and gene amplification; however, six of eight cases amplified by FISH showed positive EGFR staining. The three cases, considered amplified by CISH, also showed amplification in FISH assay, indicating a sensitivity of 37.5% and 100% specificity of CISH after receiver operating characteristic curve analysis.

DISCUSSION

Clinical trials suggest an interesting activity of EGFR inhibitors as a treatment for HNSCC.[10 11] However, some issues need to be addressed yet, as to how best to evaluate EGFR expression or whether there is a correlation between EGFR expression and patient prognosis.[12]

EGFR protein overexpression has been reported in 70–90% of HNSCC, and the incidence of gene amplification has been demonstrated in about 17–31%. Some authors have found that EGFR overexpression and amplification were associated with poor tumour differentiation and worse prognosis in HNSCC.[4 10 13–15] However, in this present study, and as demonstrated in others,[16 17]

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Table 1 Clinicopathological features, EGFR immunoexpression and CISH/FISH status of the cases

Case	Age (years)/ Gender*	Site	Tumour differentiation	T status	Nodal metastasis	EGFR expression	CISH amplification	FISH amplification	Smoking habit	Alcohol habit	Follow-up†
1	47/M	Tongue	Moderately	T1-T2	NA	−	−	−	NA	NA	NED
2	80/M	Floor of mouth	Well	NA	NA	+	−	−	NA	NA	NED
3	55/M	Tongue	Poor	T1-T2	NA	+	−	−	NA	Yes	NED
4	63/M	Tongue	Poor	T1-T2	NA	+	−	−	NA	NA	NED
5	38/M	Floor of mouth	Moderately	NA	NA	−	−	−	NA	NA	NED
6	50/M	Floor of mouth	Moderately	T1-T2	NA	+	−	−	NA	NA	NED
7	73/M	Floor of mouth	Poor	T3-T4	+	−	−	−	<	No	DOD
8	59/M	Floor of mouth	Moderately	T3-T4	−	−	+	+	<	Yes	DOD
9	40/F	Floor of mouth	Poor	T1-T2	−	−	−	−	<	Yes	NED
10	57/F	Tongue	Moderately	T1-T2	NA	−	−	−	<	No	NED
11	56/F	Floor of mouth	Poor	T3-T4	+	−	−	−	<	Yes	NED
12	44/M	Floor of mouth	Well	T3-T4	−	−	+	+	<	Yes	NED
13	45/M	Floor of mouth	Moderately	T1-T2	NA	+	−	−	NA	NA	NED
14	38/M	Tongue	Moderately	T1-T2	NA	+	−	−	NA	Yes	NED
15	62/M	Floor of mouth	Well	T1-T2	−	+	−	−	<	Yes	NED
16	54/M	Tongue	Well	T3-T4	+	+	+	+	<	Yes	NED
17	78/M	Tongue	Poor	T1-T2	NA	+	−	−	NA	No	NED
18	79/F	Tongue	Well	T1-T2	−	+	−	−	No	No	NED
19	59/M	Tongue	Well	T3-T4	−	+	−	−	<	Yes	NED
20	49/M	Tongue	Moderately	T1-T2	−	−	−	−	<	Yes	NED
21	16/F	Tongue	Moderately	T1-T2	+	−	−	−	No	No	NED
22	49/M	Floor of mouth	Poor	T3-T4	+	−	−	−	>	Yes	NED
23	80/M	Floor of mouth	Poor	T1-T2	−	−	−	−	>	Yes	NED
24	62/M	Floor of mouth	Poor	T1-T2	−	−	−	−	<	Yes	NED
25	61/M	Floor of mouth	Poor	T1-T2	−	+	−	−	>	No	NED
26	72/F	Tongue	Well	T1-T2	+	+	−	+	No	No	NED

Continued

Table 1 Continued

Case	Age (years)/ Gender*	Site	Tumour differentiation	T status	Nodal metastasis	EGFR expression	CISH amplification	FISH amplification	Smoking habit	Alcohol habit	Follow-up†
27	78/F	Tongue	Well	T1-T2	−	+	−	+	No	No	NED
28	72/F	Tongue	Poor	T1-T2	+	−	−	−	No	No	DOD
29	48/M	Tongue	Poor	T3-T4	+	−	−	−	No	No	NED
30	52/F	Tongue	Poor	T1-T2	+	−	−	−	<	Yes	NED
31	46/M	Tongue	Poor	T3-T4	+	−	−	−	<	Yes	NED
32	61/M	Tongue	Poor	T3-T4	+	−	−	−	>	Yes	NED
33	63/M	Tongue	Moderately	T1-T2	+	+	−	−	<	Yes	NED
34	69/M	Floor of mouth	Poor	T3-T4	+	+	−	−	>	No	NED
35	45/M	Floor of mouth	Well	NA	NA	+	−	−	<	Yes	NED
36	58/M	Floor of mouth	Moderately	T3-T4	+	+	−	−	<	Yes	NED
37	52/M	Tongue	Well	T1-T2	+	+	−	−	No	No	NED
38	64/M	Floor of mouth	Moderately	T1-T2	−	+	−	−	NA	NA	DOD
39	49/M	Tongue	Well	T1-T2	+	+	−	+	NA	NA	NED
40	51 / M	Gum	Moderately	NA	NA	+	−	+	NA	Yes	NED
41	40/F	Palate	Well	T1-T2	NA	−	−	−	NA	Yes	NED
42	54/M	Gum	Well	T1-T2	NA	−	−	−	NA	Yes	NED
43	44/M	Gum	Poor	NA	NA	+	−	+	NA	Yes	NED
44	51/M	Gum	Poor	T1-T2	−	−	−	−	NA	Yes	NED
45	51/M	Gum	Well	T1-T2	NA	−	−	−	NA	NA	NED
46	68/M	Palate	Poor	T1-T2	−	+	−	−	NA	No	NED
47	62/M	Gum	Well	T1-T2	NA	+	−	−	NA	Yes	NED
48	62/F	Gum	Well	T1-T2	−	+	−	−	<	No	DOD
49	44/M	Palate	Poor	T3-T4	+	+	−	−	>	Yes	NED
50	77/M	Gum	Poor	NA	NA	+	−	−	NA	NA	NED
51	53/F	Buccal mucosa	Well	T1-T2	+	+	−	−	No	No	NED
52	48/M	Gum	Moderately	T3-T4	+	−	−	−	<	Yes	DOD

CISH, chromogenic in situ hybridization; DOD, dead of disease; EGFR, epidermal growth factor receptor; F, female; FISH, fluorescence in situ hybridization; M, male; NA, not available; NED, no evidence of disease.
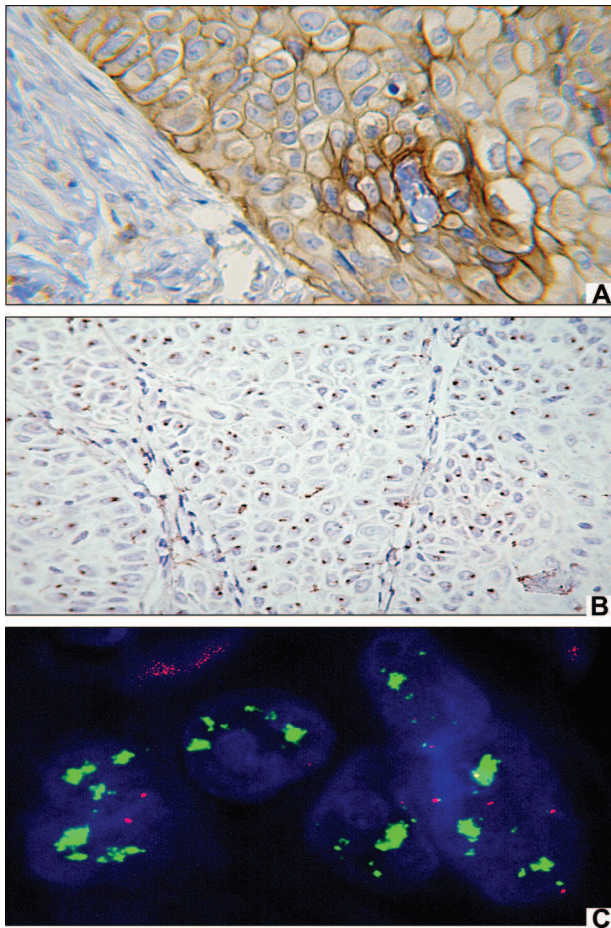*(−) negative, (+) positive, < under 20 cigarettes/day, > above 20 cigarettes/day.
†Mean 6 months.

Figure 1 Epidermal growth factor receptor (EGFR) protein expression and gene amplification in oral squamous cell carcinoma. (A) Positive staining in the membrane of neoplastic cells (×400 magnification). (B) Amplification of EGFR detected by chromogenic in situ hybridization (×400 magnification). (C) Amplification of EGFR detected by fluorescence in situ hybridization (green signal clusters represent the amplification of the EGFR gene, and red signals indicate the centromeric region of chromosome 7) (×1000 magnification).

most of the EGFR-positive lesions presented as low-grade tumours, revealing no association with patient outcome. Furthermore, EGFR overexpression has been considered a protective factor against locoregional recurrence and is related to an increased radiosensitivity.[15] Baumeister et al[18] found that high EGFR levels in normal oral mucosa render the cells less sensitive to carcinogens, and this increase might be a physiological response to permanent carcinogen impact.

In this study, we used a convenience sample, taking into account that OSCC demonstrates an increase of 42 to 58% in EGFR. In a previous study, we have already demonstrated EGFR expression in 50% of OSCC.[16] Many cases of that previous study were employed in the present one. Regarding the TMA method, Monteiro et al[8] demonstrated that the use of two 1.5-mm cores offers some advantages, such as a lower probability of sample lost, especially in heterogeneous tumours.

In addition, the authors demonstrated a strong correlation of Ki-67 and EGFR markers between the dual core TMA and the whole sections of OSCC.[8] However, in our study, we used one 1 mm core from each case in view of the small size of the incisional biopsy samples.

Szabó et al[14] give a reasonable explanation for these variable results concerning EGFR protein detection. They investigate in HNSCC samples different epitope-specific antibodies covering the entire EGFR protein. These antibodies recognised epitopes at the extracellular region close to the ligand-binding domain, membrane-proximal extracellular region, intracellular domain and the phosphotyrosine autophosphorylation site on the EGFR tyrosine kinase domain.[14] Just EGFR intracellular domain detection was associated with a worse prognosis in HNSCC. EGFR extracellular domain detection showed no clinical association. Including this study, there were various studies that did not find any association between EGFR expression and tumour behaviour[15 18 19] and used antibodies with affinity to the extracellular domain of EGFR.

High-grade tumours were frequently observed in advanced stages (T3-T4 and metastatic nodes), which however commonly revealed gene amplification, as found by Huang et al,[10] occurring in well-differentiated tumours. This incongruence might indicate that EGFR amplification has an uncertain impact in OSCC behaviour, as described by Tsiambas et al.[20]

We compared the detection of EGFR amplification by CISH and FISH techniques. Unlike other studies, we found a substantial discrepancy between these techniques. Considering FISH as the gold standard technique, CISH demonstrated a great specificity but a low sensibility, since just 37.5% of amplified FISH cases were detected by CISH. It should be due to the fact that evaluation of borderline signals could be more challenging in CISH. Its signal is highly affected by preanalytical factors in which low signal intensity or high background staining might be observed, thus impairing a correct interpretation.[21–23] This finding precludes the use of CISH as a tool for detection of EGFR gene amplification in OSCC.

Surprisingly, gene amplification correlated poorly with protein expression. Hence, as observed by others, some cases revealed protein overexpression in spite of gene amplification, as well as the inverse.[19 20 24–26] Takes et al[27] suggested that this might be the result of different methodologies to assess the status of EGFR and preanalytical issues. For Szabó et al,[14] differences in the specificity of antibodies might be responsible for it. They showed that EGFR gene amplification correlated with protein expression just when an antibody recognising the intracellular domain of the protein was used. The use of antibodies against the extracellular domains did not show any correlation. We could hypothesise that EGFR expression not only does not depend on the gene copy number, but also that the rise in protein expression does not predict specific gene deregulation. It indicates

that other mechanisms that are not subject to EGFR amplification might be involved in the rise in EGFR expression in OSCC.

We conclude that there is no association between EGFR protein expression and *EGFR* gene copy number in OSCC using the methods employed in this study. Neither EGFR overexpression nor EGFR copy number was associated with the clinicopathological characteristics of OSCC. EGFR might still be useful as a predictive marker, although for this, it is necessary to establish which is the best method to evaluate EGFR status and its relationship to response to therapy.

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