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Epidermal Growth Factor Receptor Expression in Oral Squamous Cell Carcinoma by Immunohistochemical Technique and its Correlation with Clinicopathological Features

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Abstract

Background: Oral squamous cell carcinoma (OSCC) is the most common malignancy of the oral cavity. Despite some improvements in treatment, the survival rate is still very low, mainly due to the possible development of secondary malignancy or metastasis. Clinical and pathological features as well as molecular biomarkers might predict the recurrence.

In recent years, many studies have been carried out on molecular biomarkers that can predict the prognosis of OSCC. One of these markers is the epidermal growth factor receptor (EGFR), which has led to different results. The aim of this study was to determine EGFR level in OSCC and to analyze its correlation with clinicopathological features.

Methods: A total of 62 paraffin-embedded samples from OSCC patients treated in the oncology department of the Omid Hospital in the city of Mashhad, Iran were selected and EGFR staining was performed. The clinical and histopathological data were extracted from the medical records.

Results: EGFR expression was positive in 98.4% of the cases. There was a significant difference between EGFR expression in the tumor and control cases in terms of cellularity and intensity ($p<0.001$ and $p=0.004$, respectively). No statistically significant correlation was observed between EGFR and clinicopathological parameters. There was also no significant relationship between the cellularity and intensity expression of EGFR and patient survival ($p=0.92$ and $p=0.42$, respectively).

Conclusion: In view of the high EGFR expression in squamous cell carcinoma, further studies on the role of EGFR in cell processes such as proliferation, angiogenesis and differentiation of the tumor are recommended.

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Introduction

Squamous cell carcinoma of the head and neck is the sixth most common malignancies in humans. Despite improvements in treatment in recent years, survival rates have not increased significantly (1). Different strategies are used to treat head and neck cancer, one of which is the blocking of signal transmission of some factors that affect the proliferation of tumor cells. Identifying factors that influence cell proliferation or angiogenesis is essential in finding new treatment methods.

One of the factors in cell proliferation is the epidermal growth factor, which plays an important role in the development of various types of cancer (2, 3). Some studies have been carried out on the role of EGFR in oral or head and neck squamous cell carcinoma (4, 5). It has been shown that EGFR mutation, although unusual in esophageal SCC, can be viewed as a predictor of sensitivity to anti-EGFR drugs (4). Other studies have reported a relationship between the human EGFR and the prognosis of survival rate in oral SCC patients. A study on human epidermal growth factor receptor-2 in 100 patients with esophageal cancer has shown that there is no significant difference in the overall patient survival rate between patients with and without signs of EGFR-2 overexpression (6). On the other hand, a study on oral SCC has demonstrated that there is an inverse relationship between EGFR tumor expression and patient survival (7). EGFR level could be viewed as an independent factor in predicting the survival rate (8).

In some tumors such as head and neck SCC, low EGFR expression can be associated with a high degree of differentiation and a decrease in tumor cell proliferation as well as a better prognosis (9, 10). EGFR is shown to be overexpressed in approximately 90% of head and neck SCC tumors (10).

Since there are some controversial studies on the association of EGFR expression and oral SCC in Iran and because the expression of some biomarkers is related to the race, we decided to compare EGFR expression in oral SCC with that in normal tissue among patient samples in Mashhad, Iran.

Materials and Methods

A number of 62 oral SCC samples were histopathologically evaluated at Omid Hospital in Mashhad, Iran. All blocks of oral SCC patients with available medical records were included and records with incomplete information or pathological blocks were excluded.

First, the patients' medical records were checked and the relevant clinical information such as gender, age, smoking history, as well as tumor characteristics consisting of tumor location, size and stage, type of treatment and duration of follow-up and recurrence were recorded in the checklists. The clinical stage was assessed according to the TNM system.

In terms of age, the patients were divided into four groups of 30-50, 50-70, 70-90, and $\geq$ 90 years.

After preparing the samples from pathological blocks, H&E and immunohistochemical staining was performed. For the immunohistochemical assay, slices with a thickness of 3-4 mm were made from each paraffin block and mounted on slides embedded in polyelisin. Each section was deparaffinized, rehydrated in xylene and graded ethanol, and washed in distilled water. The antigen was then obtained by incubating the sections in EDTA Tris buffer (pH = 7.5) for 30 minutes. The sections were kept in the same solution at room temperature for 15 minutes and finally, the slides were rinsed with distilled water for five minutes. The slides were incubated in hydrogen peroxide (H_2O_2, 1%) with methanol for 10 minutes to block endogenous peroxidase activity. After washing the slides in

Tris buffer, they were incubated in protein block solution for 10 minutes and washed again in Tris buffer for five minutes.

Sections were assayed for EGFR using mouse primary anti-EGFR monoclonal antibody (clone EGFR.25, code RTU-EGFR-384, 7 ml ready to use; Novocastra, Newcastle upon Tyne, UK) for 30 minutes at room temperature. The sections were then rinsed with Tris-buffered saline–tween. The Novo Link Polymer Detection System (code: RE7140-K; Leica Microsystems Inc., Bannockburn, IL., USA) was used for immuno-histochemical staining. Counterstaining was further performed using Meyer's hematoxylin followed by dehydration through soaking the sections in distilled water ethanol grades 70%, 80%, 95%, and 100%, each for five minutes, and then twice in xylene, for 10 seconds. For the control group, either normal epithelium surrounding the lesions or some lesions without epithelium involvement were selected.

The proportional staining score was the ratio of stained to unstained cells and was evaluated as in the squamous layer. It was divided into four groups according to cellularity:

1) Without labeled antibody or less than 10% labeled antibody
2) 10% ≤ labeled antibody <25%
3) 25% ≤ labeled antibody <50%
4) ≥ 50%.

To calculate the intensity score, each slide was evaluated based on the intensity of the marker in the membrane staining of tumor cells as follows:

1) 1+: weak
2) 2++: intermediate
3) 3+++: strong (11)

All samples were assessed for cellularity and intensity of staining by two pathologists who were not aware of the samples' characteristics.

Disease-free survival (DFS) was used to evaluate the patient survival and it was the time between primary treatment and follow-up visit or disease recurrence. Kaplan-Meyer and Log-rank tests were used to evaluate the survival rate. The association between each of the variables and smoking was assessed using Chi-square test. Further statistical analysis was performed using Chi-square test and Fisher's exact test as well as Kendall's tau. The statistical significance level was set to $p < 0.05$.

The study protocol was approved by the Medical Ethics Committee of Mashhad University of Medical Sciences (under the ethical code 87291). During the study, the subjects' rights and medical information were protected by the researchers.

Results

Demographic data

In the case group, 34 patients (54.83%) were male and 26 patients (41.93%) were female, whereas the data on the gender of two patients were unavailable. The patients were between 33 to 86 years old with the mean age of 60.7 ±14.77 years. In terms of smoking, 30.64% of the patients were smokers, and 53.22% had no smoking history, while the smoking history of 16.12% was unavailable (Table 1).

The evaluation of the patients' tumors revealed that 58.06% of the cases were in the tongue and 9.67% in the buccal mucosa (Table 1). Tumor grade and stage data were missed in two and 25 cases, respectively. The histopathological grade and stage of the tumors are shown in Table 1.

Table 1. The frequency distribution of studied patients based on demographic data and grade /stage of tumors

Variables		frequency	
		Number	Percent
Age (year)			
	30-50	16	25.80
	50-70	25	40.32
	70-90	19	30.64
	Data missing	2	3.22
Sex			
	Female	26	41.93
	Male	34	54.83
	Data missing	2	3.22
Smoking			
	Smoker	19	30.64
	Non-smoker	33	53.22
	Data missing	10	16.12
Location			
	Tongue	36	58.06
	Buccal mucosa	6	9.67
	Inferior labial mucosa	5	8.06
	Mandible	4	6.45
	Superior labial mucosa	1	1.61
	Inferior labial mucosa and mandible	1	1.61
	Data missing	9	14.51
Grade			
	Well differentiated	23	37.09
	Moderate	26	41.93
	Poorly differentiated	11	17.74
	Data missing	2	3.22
Stage			
	I	8	12.90
	II	6	9.67
	III	19	30.64
	IV	4	6.45
	Data missing	25	40.32
	Total	62	100

EGF analysis

There was a significant difference between the tumor and control cases, depending on the cellularity and intensity of EGFR expression, ($p<0.001$ and $p=0.004$, respectively), while tumor tissues showed a stronger staining (Table 2).

Table 2. Intensity/cellularity of staining in the tumor and control tissues

Feature	Groups	Tumor tissue		Control tissue		p value*
		Number	Percent	Number	Percent	
Intensity	Weak	1	1.63	7	14.28	P=0.004
	Intermediate	30	49.18	30	61.22	
	Strong	30	49.18	12	24.48	
	Total	61	100	49	100	
Cellularity	10-25%	1	1.63	6	12.24	P<0.001
	25-50%	8	13.11	30	61.22	
	More than 50%	52	85.24	13	26.53	
	Total	61	100	49	100	

*Exact Fissure's test

There was no significant correlation between the cellularity and intensity of EGFR expression and the mean age of the patients ($p=0.23$ and $p=0.73$, respectively), as well as their gender ($p=0.68$ and $p=0.49$ respectively). Moreover, there was no significant correlation between the cellularity and intensity of EGFR expression in tumor tissues and other variables such as smoking ($p=0.99$ and $p=0.60$, respectively), tumor location ($p=0.72$ and $p=0.84$, respectively), tumor size ($p=0.53$ and $p=0.39$, respectively), degree of histopathological differentiation ($p=0.88$ and $p=0.38$, respectively), and clinical stage ($p=0.34$ and $p=0.80$, respectively) (Tables 3 and 4).

Table 3. Staining intensity (EGFR expression) and differentiation of the tumor tissue

		Intensity of tumor tissue								p value
		Weak		Intermediate		Strong		Total		
		Number	Percent	Number	Percent	Number	Percent	Number	Percent	
Grade of differentiation	Poor	6	10	5	8.33	0	0	11	18.33	p=0.38
	Moderate	14	23.33	12	20	0	0	26	43.33	
	Well	10	16.66	12	20	1	1.66	23	38.33	
	Total	30	50	29	48.33	1	1.66	60≠	100	

≠ in two cases, data about tumor grade had been missed.

Table 4. Staining intensity (EGFR expression) and clinical stage of the disease

		Intensity of tumor tissue								
		Weak		Intermediate		Strong		Total		p value
		Number	Percent	Number	percent	Number	Percent	Number	Percent	
Staging	I	3	8.10	4	10.81	1	2.70	8	21.62	p=0.80
	II	3	8.10	3	8.10	0	0	6	16.21	
	III	10	27.02	9	24.32	0	0	19	51.35	
	IV	1	2.70	3	8.10	0	0	4	10.81	
	Total	17	45.94	19	51.35	1	2.70	37≠	100	

≠ In 25 cases, data about tumor stage had been missed.

Sufficient information about follow-up sessions was included in 79% of patient records, and the mean follow-up was 17.3±18.57 months. The relationship between the EGFR intensity and disease-free survival was evaluated in patients who had been prescribed a treatment protocol using the Kaplan-Meyer and Log-rank tests. The analysis showed no significant relationship between the cellularity and intensity expression of EGFR and the patient survival (p=0.92 and p=0.42, respectively).

In addition, there was no significant correlation between the survival rate and the degree of histopathological differentiation as well as the clinical stage of the tumor (p=0.41 and p=0.81, respectively).

After analyzing EGFR overexpression in patients with and without recurrence, no significant relationship was found between the cellularity and intensity expression of EGFR and the recurrence rate (p=0.35 and p=0.99, respectively), as well as between the degree of histopathological differentiation and the clinical stage of the tumor as well as the recurrence rate (p=0.13 and p=0.99, respectively).

The patient's survival rate had no significant relationship with the tumor size, tumor location and treatment protocol (p=0.56, p=0.22 and p=0.67, respectively).

The analysis showed no significant relationship between the survival rate and demographic information such as age and gender (p=0.20 and p=0.05, respectively). There was also no significant difference between the survival of smokers and non-smokers (p=0.75).

Discussion

In this study, there was a significant difference between SCC samples and control tissues in terms of the cellularity and intensity expression of EGFR; although, no positive correlation was observed between EGFR expression and clinical, as well as histopathological parameters.

Finding out the role of molecular factors and their correlation with clinical manifestations is beneficial in determining the prognosis and proper treatment of various types of cancer. Therefore, the development of new therapeutic modalities, mainly targeted therapies based on an understanding of the biology and molecular factors in cancer, could lead to a better response to treatment and an improvement in the quality of life of SCC patients (12).

Some studies have suggested different biomarkers in head and neck cancer such as EGFR. However, reports on EGFR appear to be inconsistent; in some studies, the EGFR rate has been reported to be increasing in cancer patients, but others have reported no change (13, 14). Studies have also shown that although there has been an EGFR overexpression in 33-50% of epidermal tumors, overexpression is observed in more than 90% of head and neck tumors (15, 16).

In this study, EGFR protein expression was evaluated through immunohistochemical staining, and it was positive for 98.4% of patients' samples. This was consistent with other studies indicating EGFR overexpression in most of head and neck, as well as oral SCC samples (17-20).

In another study conducted in the city of Tehran, Iran, EGFR overexpression was reported in 65% of esophageal SCC samples (4), which was consistent with our results, where overexpression was detected in most of the cases. It seems that the expression of these factors is different in various types of tumors in different parts of body.

There are also some differences between EGFR RNA and protein overexpression. In Gonzaga *et al*. study, 11% of ESCC tumors showed an increase in EGFR mRNA levels compared to normal mucosa, while only 4% showed protein overexpression (21). In contrast, in Sunpaweravong *et al.* and Hanawa *et al.* studies, protein overexpression was found in more than 40% of ESCC patients, while gene amplification has been reported in 15% of the cases (22, 23).

Some authors believe that such different results may be due to different methods to assess EGFR staining by Immunohistochemistry (21).

Activation of EGFR appears to promote cell migration and invasion by producing MMP-9, followed by the breakdown of E-cadherin (24).

Other studies on head and neck SCC have shown that immunohistochemical staining of tumors is more intense compared to that of normal epithelium and that poorly differentiated tumors show more severe staining than good and moderate differentiated SCC (1, 25). In contrast, some studies have reported that there is a direct relationship between EGFR overexpression and good differentiation of tumor cells (26, 27). Howover, other studies have found no association between cell differentiation, clinical properties and EGFR expression(10, 28), which is consistent with our results.

Previous studies have also shown a correlation between EGF expression and the clinical properties of the tumor. Chuang *et al*. has demonstrated that there is a direct correlation between the EGFL6 plasma levels and tumor size, distant metastasis, and tumor stage (29).

It has been also confirmed that EGFR overexpression is associated with an increase in tumor size or features such as lymph-node metastases and patient survival since EGF induces migration in connective tissue-derived cells (30-32). In this study, however, there was no association between EGFR expression levels and the clinical stage of tumor. There was also no relationship between EGF level and tumor size, lymph-node involvement, and metastasis. These results were consistent with other studies in which there was no connection between EGFR overexpression and clinical, histopathological, biological and prognostic properties (17, 28, 33); however, there has been reported a direct correlation between expression of this factor and tumor invasion (17). In addition, despite positive immunohistochemical staining in 87.5% of oral SCC patients

in a study by Sarkis *et al.*, it was not associated with clinical parameters. These results might imply that this marker is independent of tumor behavior (34).

Moreover, conflicting results on the role of EGFR on disease prognosis have been reported in some previous studies. Some studies have stated that EGFR overexpression found in most cases of head and neck SCC is associated with a poor prognosis (19, 35-37). In contrast, Kanematsu *et al.* have demonstrated that while EGFR phosphorylation is associated with a poor prognosis, EGFR overexpression does not predict a poor prognosis for oral SCC (38).

Moreover, Gao *et al.* have shown that the number of overexpressed EGFR ligands is related to five-year survival, even in patients with advanced stage of IV; even though, these ligands did not mediate cisplatin resistance in the OSCC cell lines (39).

In our study, EGFR level was not related to prognosis, response to treatment, and survival. But, Laimer *et al.* have suggested that EGFR overexpression predicts poor prognosis in patients with oral and oropharyngeal SCC (19). Smid *et al.* have shown that there is no association between EGFR expression and treatment or survival (40).

Yarden *et al.* have reported that among ten types of cancer, in the head and neck cancer, EGFR level has a high prognostic value (41).

Some studies have indicated a relationship between the response to radiotherapy or chemotherapy and the EGFR level. Zhao *et al.* have found that EGFR predicts radio sensitivity and prognosis in squamous cell carcinoma of the human esophagus (42).

It appears that the different findings on the relationship between EGFR level and clinical parameters could be due to race, gender and age. In fact, such conflicting results could be due to different factors in survival and carcinogenic pathways that differ among populations.

In our study, tumor stage and grade showed no relationship with survival, probably due to short-time follow-ups, especially for tumors with high grade and stage. In contrast, Lo *et al.* have stated that not only the degree and stage of tumor, but also tumor size, lymph node involvement and metastasis as well as smoking can affect patient survival (43). On the other hand, gender and age showed no relationship with survival in this study. Similar to our results, another study has concluded no correlation between gender and patient survival (44); whereas, Chen *et al.* have reported a lower survival rate in women, which could be due to late referral to physician or reluctance to treatment in some women (45). Paradoxical results could be due to differences in cultures and socio-economic conditions that affect referral to a physician.

Furthermore, some cancer risk factors may be related to EGFR level; even though, we found no association between smoking and patient survival.

In short, the association of EGFR level with clinical and pathological parameters appears to be controversial in different populations and requires further studies for more reliable results.

Conclusion

Although EGFR was positive in most of the oral SCC patients in this study, EGFR level showed no correlation with tumor size, location and clinocopathological characteristics, as well as patient survival. Therefore, further studies with larger sample size and molecular tests are recommended.

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