Bio Functional Evaluation of EGFR Expression as a Prognostic Indicator in Oral Squamous Cell Carcinoma and a Recommendation for Personalized Targeted Therapy with Anti EGFR Inhibitors

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Abstract

Background: Carcinoma is a complex, multifactorial and a devastating disease. Understanding the molecular changes that leads to disease enables one to develop strategies to prevent, provide an early diagnosis and serve as targets for therapy. Epidermal Growth Factor Receptor (EGFR), a 170-kDa cell-surface protein is a tyrosine kinase receptor from the ErbB family and is involved in many biological processes that affect tumour development, growth progression, differentiation, inhibition of apoptosis and development of metastasis. Methods: Study included 54 cases of oral squamous cell carcinomas. EGFR expression by immunohistochemistry has been studied, correlated with clinical parameters, known prognostic factors and patient survival to note its significance.

Conclusion: Head and Neck Squamous Cell Carcinoma (HNSCC) with or without regional lymph node metastases, are challenging to treat effectively. The prognosis for the patients with advanced stage of the disease still remains poor. The correlation of EGFR overexpression with tumour size, grade, stage and survival suggests that EGFR expression can be considered as having prognostic significance and is a potential target for therapy with EGFR inhibitors in HNSCC. Further studies are required to assure the viability of EGFR levels in saliva as non-invasive prognostic marker and also as an indicator of recurrence.

Keywords*: Epidermal Growth Factor Receptor (EGFR), EGFR inhibitors Immunohistochemistry, Squamous Cell Carcinoma.*

Introduction

Carcinoma is complex, multifactorial and devastating disease.[1] HNSCC is ninth most common carcinoma in the world.[2]Oral cancers accounts for one third of the global burden of cancers which depends on the exposure to major risk factors like tobacco smoking, chewing areca nut and alcohol consumption.[3]Understanding the molecular changes that leads to the disease enables one to develop strategies to prevent, provide an early diagnosis and serve as targets for therapy.[1] HNSCC generally develop through accumulation of multiple genetic and epigenetic alterations in multistep process which could include deletion or methylation of tumour suppressor regions, mutations, amplification or overexpression of oncogenes. Some of these alterations occur in early tumorigenesis and are identified in tissues that are chronically exposed to common carcinogens and in premalignant lesions of head and neck.[4] In recent years mutation of p53, over-expression of EGFR, cyclin

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D1, Bcl-2 and transforming growth factor (TGF-alpha) are found to be of prognostic importance.[5] EGFR, 170-kDa cell-surface protein is a tyrosine kinase receptor from ErbB family and is involved in many biological processes that affect tumour development, growth progression, differentiation, inhibition of apoptosis and development of metastasis.[4,] Verma *et al*[6]., have observed overexpression of EGFR in 40%–80% of head and neck cancers.[6,7] Its overexpression is associated with malignant phenotype and results in poor prognosis.[8] Studies have prompted development of clinical trials testing EGFR inhibitors as single-agent therapy or in combination with conventional cytotoxic therapy.[9]The present study aims to establish the prognostic value of EGFR expression in oral cancers which could likely benefit patients with the use of EGFR inhibitors and to study the expression of EGFR in oral squamous cell carcinomas and to correlate it with prognostic factors that could guide initiation of anti-EGFR therapy.

Materials and Methods

It was a retrospective and a prospective in-vivo, observational study of EGFR expression in 54 oral squamous cell carcinomas undertaken at JSS Dental College and Hospital in Department of Oral Medicine and Radiology, Department of General Pathology, JSS Medical College, JSSAHER, Mysuru from year 2018 till 2019 with the approval of institutional ethical committee. The formalin fixed paraffin embedded sections were studied for various morphological parameters and subjected to Immunohistochemistry (IHC) staining with EGFR and the expression was correlated with clinical parameters, known prognostic factors and patient survival.

Immunohistochemical staining: 3 - 4 µm thick sections were fixed on Poly-L-Lysine coated slides, deparaffinised and rehydrated. The sections were incubated with primary antibody EGFR (DAKO, FLEX Monoclonal Mouse Anti-Human EGFR), for 30 minutes, washed with wash buffer and further incubated with labelled polymer – HRP (DakoEn Vision + Dual Link System – HRP, DAB+, Code K4065). The bound antibodies were visualized using a DAB-chromogen, counterstained with haematoxylin and mounted with DPX. The staining pattern, the percentage of stained cells and the intensity of the stain was evaluated by two independent observers.

Evaluation of EGFR staining pattern: To quantify EGFR expression a total immunostaining score was calculated, using the product of a proportion score and an intensity score. The proportion score described the estimated fraction of positive stained tumour cells (0 = none; 1 = <10%; 2 = 10–50%; 3 = 50–80%; 4 = >80%). The intensity score represented the estimated staining intensity (0, no staining; 1, weak; 2, moderate; 3, strong). The total score obtained by multiplying the two scores and it ranged from 0 to 12. Tumours were categorized into samples with high EGFR, low EGFR and no EGFR expression using a cut-off value of: 0 = none, 1–3 = low, 4-12 = high according to Laimer*et al*.[5]

Statistical methods: The data was represented in terms of frequency distribution tables. The proportion of subjects revealing the expression according to the various subgroups such as pathological staging, age, location and lymph node status was estimated along with 95% confidence interval. The possible associations between EGFR overexpression, treatment outcome, and other categorical variables were evaluated with Fisher's exact test. Association of continuous variables was evaluated with t-test. The log-rank test was done to compare survival among groups of patients. Survival curves were generated by the univariate and multivariate analysis to get Kaplan-Meier survival curves. A significance level of .05 was used for all statistical tests.

Results and Discussion

In the present study, majority (56%) of the patients were males with a male to female ratio of 1.85:1 and the male patients showed higher EGFR expression. The mean age of presentation was 52 years and age range were between 30-75 years. EGRF expression was predominantly of high grade in patients above 40years. The tumours were seen predominantly involving the tongue (63%) (Figure 1), while the remaining cases were seen to involve the floor of the mouth (15%) (Figure 2), retromolar area (11%) and the posterior palate (11%).

Figure 1: Carcinoma of tongue

Figure 2: Carcinoma of floor of the mouth

Correlation of EGFR expression with sex and age distribution and site of involvement showed no significant correlation (0.109). There was no significant family history among the subjects included in the study. 74% of patients were exposed to some form of tobacco and they showed increased EGFR levels compared to subjects not exposed to either forms of tobacco. 37% of cases were exposed to alcohol while the remaining 63% were not exposed to alcohol, the EGFR expression in both the groups showed no significant difference. On gross examination 14 cases were of <2cms and 40% of these cases showed low EGFR staining. The 28 cases which were of 2-4 cms and 12 cases which were >4cm showed high EGFR positivity. The intensity of EGFR staining of the tumour cells became stronger with increase in tumour size, with the 'p' value showing statistical significance (0.0001). On grading 56% of cases were moderately differentiated, of which majority (96%) showed high EGFR scores (Figure 3). 30% of cases were well differentiated of which few (17%) showed low EGFR score (Figure 4) and remaining 14% were poorly differentiated and all of which showed high EGFR score.

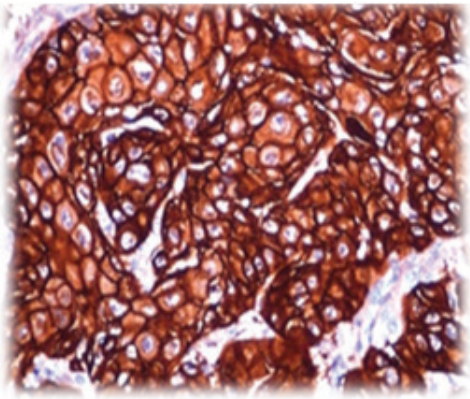

Figure 3: High grade

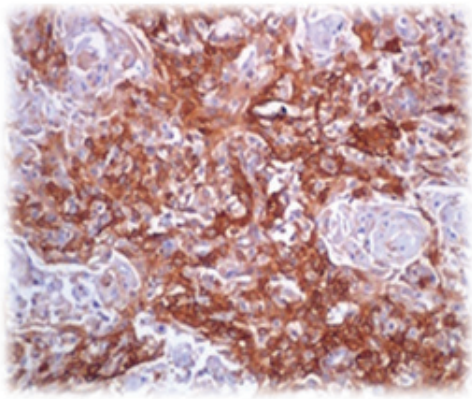

Figure 4: Low grade

Majority of tumours were of stage II and III and there were equal number of stage I and IV tumours. Stage III tumours showed nodal involvement in only 40% of cases. In stage IV tumours 56% of cases had metastasis in single ipsilateral lymph node which was >3cms but <6cms in its greatest dimension, 33% of cases showed multiple ipsilateral lymph node involvement, while only one case showed a single node involvement of >6cms. 30% of tumours of stage I showed low EGFR score. 95% of stage II, and 100% of stage III and IV tumours showed high EGFR levels and this was statistically significant. Survival was better among tongue tumours, with no history of exposure to tobacco, better differentiation, lower TNM staging and low EGFR levels. (Table 1)

Table: 1 Correlation of EGFR expression levels with prognostic factors and survival.

Variables		EGFR Low	EGFR High	Survival (%)
Site	Tongue	03	31	88%
	Floor of mouth	00	08	60%
	Retromolar region	00	06	
	Posterior palate	01	05	
History of Tobacco	Negative	00	14	100%
	Positive	04	36	76%
Grade	Well Diff	03	14	100%
	Moderately Diff	01	29	80%
	Poorly Diff	00	07	25%
TNM Staging	Stage I	03	07	100%
	Stage II	01	21	97%
	Stage III	00	12	93%
	Stage IV	00	10	11%
EGFR levels	Low	04	00	100%
	High	00	50	66%

HNSCC are one of the most leading cancers in India and the incidence is high compared to the rest of the world.[10] EGFR is a tyrosine kinase transmembrane cell surface receptor with an extracellular ligand binding domain and an intracellular tyrosine kinase domain and mediates its mitogenic effect by binding to the cell-surface receptor.[8,11] It is highly expressed in several human tumours and contributes to the pathogenesis of disease. Increased levels of EGFR expression, as detected by IHC have been shown in up to 90% of HNSCC tumours.[12] The high expression of EGFR correlates with a more aggressive clinical course and has been reported to be a useful diagnostic and prognostic marker. In recent years, it has also been considered a promising target for monoclonal antibody therapy.[13]

Age has been recognized as a prognostic factor for tumour recurrence, the risk of head and neck cancers (HNC) increases with age and most oral cancers occur in people older than 40years as seen in the present study. The age distribution is consistent with other studies.[14, 15] and shows significant correlation with EGFR expression (0.004). Men in most countries have a two to five-fold greater risk of HNC than women, this finding is substantiated by the male predominance seen in the present study with high EGFR expression. This difference is likely related to higher rates of substance abuse, particularly tobacco use, among men.[16]The tumours were seen predominantly to involve the tongue with lateral border being most common site. There was no significant difference in EGFR expression with site of involvement and this was consistent with other studies.[14, 15]

Approximately 90% of patients with HNC have a history of tobacco use, tobacco smoke contains 5,000 chemicals including polycyclic aromatic hydrocarbons, N-nitrosamine's and Azarenes, which have shown to possess antigenic, cytotoxic, mutagenic, carcinogenic and genotoxic properties. These chemicals induce DNA adducts, mainly 6-methyl-guanine which interfere with DNA replication throughout the genome and can damage proliferating cells.[17]In the present study there was a predominance of tobacco users which was similar to that seen in previous study by Maiti GP *et a.l*[18] and there was a statistically significant correlation of smoking with EGFR over expression. Zhou J *et al*[19], in his study stated that long-term use of smokeless tobacco increases the risk of HNSCC.[19] Rettig *et al*[17]. in their study have concluded that tobacco in both smoked and smokeless forms is most important risk factor for both development and prognosis of HNSCCs.[17] Studies have also shown a dose-response effect between duration of smoking and increased risk of cancer and risk reduction after cessation of smoking.[20, 21]In the United States 7% of oral cavity cancers are attributable to chewing tobacco including betel quidor and areca nut, while it is as high as 53% in India and 68% in Sudan.[17]Alcohol is second to smoking as etiological factor in development of HNSCC and it is difficult to distinguish the separate effects of alcohol and smoking.[22]The risk of developing cancer in patients who both smoke and consume alcohol has a multiplicative effect rather than an additive risk.[23]Alcohol is not carcinogen, but acetaldehyde, the first ethanol metabolite produced in gastrointestinal tract by alcohol dehydrogenase (ADH) enzymes forms DNA adducts that interfere with DNA synthesis and repair, this has been shown to be toxic, mutagenic and carcinogenic.[24]In the present study the EGFR expression in alcoholics and non-alcoholics showed no significant difference.

As seen in the present study, Chen *et al* [25]and Santini *et al* [26]have demonstrated a positive correlation between the tumour size and EGFR expression. Majority of tumours of 2-4cms and all cases with size >4cm, showed high EGFR expression. The intensity of EGFR staining of tumour cells became stronger with increase in tumour size, with 'p' value showing statistical significance (0.012).

HNSCC is graded according to Broder's classification histologically into well, moderately, or poorly differentiated tumors.[4] Moderately differentiated tumours were more predominant in the present study and this observation was consistent with Laimer *et al*[8], Rodrigo *et al*[27]and Scambia *et al*[28], while Verma et al,[6] Vats *et al*[14] and Zafar *et al*[15] have found predominantly well differentiated tumours. There was a statistically significant association of EGFR expression with tumour grade (0.02) in the present study and most studies have confirmed similar findings.

The most frequently used WHO system for staging cancer is the TNM staging systems, the importance of which includes an indication of patient prognosis, a

guide for treatment choice, a comparison of treatments in same stage patients and a facility for the exchange of information between clinicians.[29] In the present study all tumours of stage I and 47% of stage II showed low EGFR score, while 57% of Stage II, and all tumours of Stage III and IV showed high EGFR scores and it was noted that EGFR scores increased with increasing TNM staging and it was found to be statistically significant (0.02).

There was significant difference in survival in favor of patients who had lower proportional EGFR score of 0 to 2 compared to higher scores of 3 and 4 (0.002) which indicates that patients with higher EGFR expression have poorer prognosis compared to patients with lower EGFR expression. Similar results were seen in the study by Laimer *et al*[8]., who has found high EGFR expression correlating significantly (0.05) with poor survival.[8] Survival analysis has shown better survival in patients <40years, with tumours <4cms, with no history of exposure to tobacco, better differentiation, lower TNM stage and low EGFR levels.

Conclusion

The correlation of EGFR overexpression with tumour size, grade, stage and survival suggests that EGFR expression can be considered as having prognostic significance and is a potential target for therapy with EGFR inhibitors in HNSCC. These findings need to be verified on larger cohort for further consideration and further studies are required to assure the viability of EGFR levels in saliva as non-invasive prognostic marker and also as an indicator of recurrence.

Ethical Clearance – Taken from Institutional Ethical Committee – Number: 5813

Source of Funding – Self

Conflict of Interest – Nil

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