

Correlation of clinicopathological parameters with EGFR and Her-2 neu status in oral cancer patients in a tertiary care centre

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Abstract

Background: Oral squamous cell carcinoma (SCC) is one of the most frequent cancers in the world. The overall postoperative survival has not improved much despite advanced surgical techniques and anticancer drugs. A better understanding of molecular mechanisms and identification of potential oncogenes in oral carcinomas may provide new therapeutic decisions such as target therapy in the treatment of these patients.

Aims & objectives: The present study aimed at analyzing the clinicopathological profile of patients with oral SCC and also, to determine the levels of expression of EGFR and Her-2 neu in these lesions and carry out their correlation with the clinicopathological parameters.

Material and Methods: The expression of EGFR & Her-2 neu was studied in paraffin embedded tissue sections of oral SCC after taking a detailed history and examination.

Results and Conclusion: EGFR was positively expressed in 84.4% (38/45) of squamous cell carcinomas and 79% (19/24) of dysplasias. While Her-2 neu was positively expressed in 62.2% (28/45) of carcinomas and 66.7% (16/24) of dysplasias. The correlation between tumor differentiation and immunomarker expression came out to be significant for EGFR but not Her-2 neu. Thus, a high level of EGFR is strongly associated with tumor aggressiveness. Molecular targeting of EGFR can therefore, is used for adjuvant treatment in malignancy.

Keywords: Oral, squamous cell carcinoma, EGFR, Her- 2 neu

1.Introduction

Worldwide, oral cancer accounts for 2%–4% of all cancer cases [1]. In India, it ranks number one in terms of incidence among men and third among women [2]. Despite the advances of therapeutic approaches, percentages of morbidity and mortality have not improved significantly during the last 30 years [3].

The standard treatment for patients with this cancer is surgery and radiation but this is far less successful in patients with advanced carcinoma. Though clinical staging does help in treatment planning and prognosis estimation, it does not provide enough predictive information right at the onset of disease process which would benefit the patient. Therefore, the search for different prognostic markers still remains [4]. The tyrosine kinase receptor, epidermal growth receptor (EGFR) family proteins EGFR and Her-2 have been reported to be over expressed in many cancers. They are often associated with increased tumour size, grade, metastasis, shortened survival and hence a poor prognosis, suggesting that they are potential molecular targets for anticancer therapy [5].

Aligarh (India), our district of study has majority of its population consuming tobacco, betel nut and alcohol which makes oral cancer a major health issue here. Hence in the light of these aspects, the present study was taken up to assess the clinicopathological profile of patients with squamous cell lesions of the oral cavity, evaluate the expression of EGFR and Her-2neu and sought possible correlation with tumour parameters.

2. Material and methods

Two hundred fifty one patients with biopsy proven squamous cell dysplasia and carcinoma of the oral cavity who presented in various O.P.Ds of J.N Medical College and Hospital, Aligarh, India were included in the study.

The biopsy fragments were fixed in 10% formalin and processed by histopathological technique by paraffin embedding and Hematoxylin-Eosin stain. The epidemiological and histological data were analyzed and the lesions were classified according to the latest WHO criteria. Subsequently, serial sections were performed and subjected to

immunohistochemical analysis using rabbit antihuman polyclonal antibodies.

2.1 Staining procedure: Immunohistochemistry

Sections, 3µm thick, were mounted on positive charged microscope slides. After dewaxing in xylene, sections were dehydrated in ethanol, rinsed in distilled water, placed in 3% H₂O₂ for 10 min and rinsed in distilled water for 15 min for antigen retrieval procedure. Slides were placed in citrate buffer solution, PH=6, in microwave at 92°C for 10 min. After cooling at room temperature for 20 min, slides were exposed to primary antibodies (BioGenex polyclonal rabbit anti-human EGFR antibody AR335-5RE; Clone EP38Y IgG1, BioGenex monoclonal mouse anti-human HER-2/neu Receptor antibody; Clone CB11; IgG1) were used (Diluted in PBS, pH 7.6, containing 1% BSA and 0.09% sodium azide) in dilution of 1:10-20. Sections were washed again and incubated with secondary antibody for 30 min followed by the streptavidin biotin- peroxidase (strept ABC complex, HRP duet kit, Dako) for 30 min at room temperature. Reactions were developed with a solution containing 0.6 mg/mL of 3,3'-diaminobenzidinedihydrochloride (DAB, sigma) and 0.01% H₂O₂ and counter stained with Mayer's hematoxylin for about 2 min.

The validation of the reaction was achieved by using negative external controls, with primary antibody omission and below mentioned positive controls.

Her2neu: Tumor epithelial cells of invasiveductal carcinoma of breast

EGFR: Basilar squamous epithelium of cervix.

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*[6] 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).

Her2/neu reactions were quantified based on a system with four degrees, according to the number of marked cells and reaction intensity [7]:

- 0 – absence of the reaction or membrane reaction, in less than 10% of the cells;
- 1+ – weak or incomplete reaction, in more than 10% of the cells;
- 2+ – weak or moderate and complete reaction, in more than 10% of the cells;
- 3+ – intense and complete reaction, in more than 10% of the cells.

These scores were subsequently grouped into two categories: nil/low expression (0 or 1) and high expression (2 or 3).

The statistical analysis was done using Fisher's exact test. Differences were considered significant when $p < .05$. The analyses employed SPSS 10 software.

3. Results

3.1 Clinicopathological Results

Of the 251 patients, 48 were dysplasias and 203 were malignancies (Fig. 1). According to the gender wise distribution, 178(71%) patients were males and 73(29%) were females. Of malignant cases, 144 were males and 59 were females. Maximum cases of both dysplastic and malignant cases were present in the age group of 41-50 years (72/251) followed by 31-40 years (58/251). The average age of males and females was 50.24 and 50.13 years respectively. The commonest site was found to be buccal mucosa (BM-47.4%) followed by tongue (23.1%), and alveolus (9.2%). The most common identifiable risk factor was tobacco smoking followed by tobacco chewing and betel quid consumption. Of 48 premalignant lesions, 22 patients had mild, 16 patients had moderate and 10 patients had severe dysplasia. 114 patients had well differentiated (WDC), 63 had moderately differentiated (MDC) and 26 patients had poorly differentiated carcinoma (PDC). (Fig. 1)

Of the 203 patients, 126 patients (62.4%) presented with lower tumor size (T1+T2) and 152 patients (75%) presented with lower nodal status (N0+N1). On the other hand, 77 patients (37.6%) presented with higher tumor size (T3+T4) and 51 patients (25%) presented with higher nodal status (N2+N3). Accordingly, 85 patients (42%) had early stage disease (Stage I+II) while 118 patients (58%) had late stage disease (Stage III+IV).

3.2 Immunohistochemistry Results

Out of a total of 251 cases, immunohistochemistry was applied to 69 cases which were randomly selected and comprised 24 cases (50%) of dysplasia and 45 cases (25%) of SCC. The pattern of the immunoreaction was a distinctive brown staining in the cytoplasmic membrane of the neoplastic cells. The immunoreactions analysis for EGFR indicated positive results in 19 of 24 patients (79%) with dysplasia but no correlation with the severity of dysplasia was seen. 4 cases each of moderate and severe while 2 cases of mild dysplasia showed high expression of EGFR (Table 1)(Fig. 2a,b). On the other hand, 9 cases of mild, 4 of moderate and 1 case of severe dysplasia exhibited low expression.

In 45 patients with carcinoma, EGFR showed positivity in 84.4% cases. Out of 22 cases of well differentiated carcinoma, 6(27.3%) showed high expression (Fig. 2c) while 16 cases (72.7%) showed low expression of EGFR. On the other hand, 15 of 23 cases (65.2%) of the lesser differentiated forms showed high expression (Fig. 2d) while 8 cases (34.8%) showed low expression (Table 2). The correlation between EGFR expression and tumor differentiation and various other tumor parameters like tumor size, nodal status and stage of the tumor was found to be statistically significant (Table 3).

Her-2 neu protein expression was found in 16 cases of dysplasia (66.7%). 18% (2/11) cases of mild, 50% (4/8) of moderate and 60% (3/5) of severe dysplasia showed high expression (Fig. 3a,b) while 82% (9/11), 50% (4/8) and 40%

(2/5) cases respectively showed low expression (Table 2;). No significant correlation was seen between the severity of dysplasia and Her-2 neu expression.

The pattern of Her-2 neu expression in carcinomas was as follows: 31 cases of 45 (65%) exhibited positivity. Of a total of 22 well differentiated cases, 5 cases (22.7%) showed high expression (Fig. 3c) whereas 17 cases (77.3%) showed low expression. On the other hand, 9 of 23 cases (39.1%) of the lesser differentiated forms showed high expression (Fig. 3d) while 14 cases (60.7%) showed low expression (Table 2). The correlation between Her-2 neu expression and tumor differentiation as well as other tumor parameters was not found to be statistically significant (Table 3).

4. Discussion

EGF receptors are members of the EGF growth factor receptor tyrosine kinase family and are generally found on the cell surface. The receptor for the epidermal growth factor (EGFR) is 170–180 kD transmembrane glycoprotein tyrosine kinase receptor identified in more than one types of cancer, including breast cancer, prostate cancer, pulmonary cancer, bladder cancer, head and neck cancers including oral cancer [8]. In the present study, we found varying degrees of expression in oral dysplasias: 79% (19/24) and SCCs: 84.4% (38/45). Literature facts also confirm as well the EGFR overexpression in over three quarters of the oral SCC analyzed cases [9-11].

EGFR expression involved all epithelial layers in oral SCC specimens while in normal oral epithelia it was localized to the basal cell layer, as documented in other studies. [12,13]. EGFR expression extent and intensity scores revealed by studies suggest that EGFR expressing carcinomas display pathological features of more aggression which may be attributable to the activation of different signaling pathways that control diverse biological processes [11,14]. In the present study as well, a statistically significant correlation was found between the EGFR expression, tumor size, node status and stage of the tumor while no correlation was observed in cases of dysplasia. A series of reports have shown a connection between the EGFR expression and the resistance to ionizing radiation [15,16]. *In vivo*, inhibition of EGFR by a monoclonal antibody, cetuximab, increases the response of squamous carcinomas to irradiation. [17]. On the contrary, studies done by Bernardes *et al* and Yamada *et al* found the EGFR expressing carcinomas to be well differentiated in most of the cases. These studies documented those EGFR-positive lesions presented as low-grade tumours, revealing no association with patient outcome [18-20]. Furthermore, Smith *et al* emphasized on the fact that EGFR overexpression had a protective role against loco regional recurrence and is related to increased radio sensitivity [21]. Therefore, the EGFR antigen represents an attractive target for specific therapies using monoclonal antibodies or tyrosine kinase inhibitors to these patients [10,14].

The oncoprotein EGFR2 (HER2/neu) is a ~185KD tyrosine kinase transmembrane receptor that belongs to the same family as epidermal growth factor receptor. Its over expression was observed in multiple types of cancer and used in treatment measures against cancer. It has been proved that the Her-2 neu over expression increases the metastatic potential, by promoting invasion through multiple stages and the metastatic cascade, suggesting that this gene might play an important role in carcinogenesis. However, the purpose of the Her-2 neu in oral squamous carcinomas is not well defined [22].

The analysis of the Her-2 neu expression in the present study indicated a very aberrant positivity in 66.7% (16/24) cases of dysplasia and 62.2% (28/45) cases of oral SCCs. There was no correlation between percentage of Her-2 neu staining and tumor differentiation. Literature studies concerning Her-2 neu in oral SCCs communicated the protein over expression in a small number of tumors and it does not appear to have any importance in prognosis [11,18,23,24]. On the other hand, Xia *et al* reported Her-2neu to be the most significant single factor in predicting disease outcome [25].

Conflicting results in different studies might be due to using different immunohistochemical methods (direct, indirect), type of antibody (clone CerbB2, CB11, ICR1b, polyclonal DAKO, monoclonal zymed) no specific criteria for positive staining of Her-2 neu protein (Membrane and/ or cytoplasmic) and /or using different techniques (immunosorbent assay, radioimmunoassay, IHC) or different locations of lesions and sex of patients with oral SCC [23].

Taken together, our results demonstrate that EGFR over expression is statistically associated with lesser differentiation, tumor aggressiveness, tumor stage, LN status, overall TNM stage and poor survival. It can therefore, serve as a good prognostic indicator and a target for anticancer therapy. Moreover, the simplicity of determining EGFR status, by an immunohistochemical technique, suggests that it could be applicable in diagnostic strategies together with the other prognostic factors that indicate therapeutic integration and more careful follow-up. On the other hand, no significant association was observed between the aforementioned tumor parameters and Her-2 neu expression. Hence, its role as a prognostic indicator remains controversial.

In recent years, EGFR has been considered a promising target for monoclonal antibody therapy [26,27]. It is of great importance to conduct studies to determine the spectrum of mutations in the human EGFR 2 gene in order to gain a better insight into the mechanisms responsible for the over expression of this frequently activated biomarker in human oral cancer. Oral tumors over expressing EGFR exhibit a higher proportion of complete responses to chemotherapy than tumors with low-level EGFR expression. Over expression of EGFR presumably due to higher intrinsic proliferative activity could result in higher sensitivity to drug therapy cytotoxic to cells undergoing mitogenesis [28]. Numerous *in vivo* experiments have shown their anti tumor activity against

tumor cell lines through a range of mechanisms, including an antiproliferative effect, direct cytotoxicity, and the potentiation of the cytotoxic effects of chemotherapy or radiotherapy [29]. Although there has been considerable

development in this field in India as well, it still needs more research and validation in a larger population. This study aims at getting a better understanding of the role of growth factor receptors in oral tumorigenesis in North Indian population

Table1: EGFR and Her 2-neu expression in dysplastic squamous lesions

Type	EGFR expression		Her 2-neu expression	
	Nil/Low	High	Nil/Low	High
Mild dysplasia (n=11)	9 (81.9%)	2 (18.2%)	8 (81.8%)	3 (18.2%)
Moderate dysplasia(n=8)	4 (50.0%)	4 (50.0%)	3 (50.0%)	5 (50.0%)
Severe dysplasia (n=5)	1 (20%)	4 (80%)	2 (40.0%)	3 (60.0%)
Total (n=24)	14 (58.3%)	10 (41.7%)	15 (62.5%)	9 (37.5%)

Table2: EGFR and Her 2-neu expression in oral squamous cell carcinomas

Type	EGFR		Her 2-neu	
	Nil/ Low	High	Nil/Low	High
WDC(n=22)	16 (72.7%)	6 (27.3%)	17 (77.3%)	5 (22.7%)
MDC (n=16)	6 (37.5%)	10 (62.5%)	11(68.8%)	5(31.2%)
PDC (n=7)	2 (28.6%)	5 (71.4%)	3 (42.9%)	4 (57.1%)
Total (N=45)	24 (53.3%)	21 (46.7%)	31(56.4%)	14 (31.1%)

Table 3: Analysis of the association of clinicopathologic covariates with EGFR and Her 2-neu expression in oral squamous cell carcinoma

Tumor parameter	EGFR expression		Her 2-neu expression	
	Nil/ Low	High	Nil/ Low	High
Differentiation: Well (n=22)	16 (72.7%)	6 (27.3%)*	17 (77.3%)	5 (22.7%)
Mod+poor (n=16+7)	8 (34.8%)	15 (65.2%)*	14 (60.7%)	9 (39.1%)
Tumor size: T1+T2 (n=28)	19 (67.9%)	9 (32.1%)*	21 (75%)	7 (25%)
T3+T4 (n=17)	5 (29.4%)	12 (70.6%)*	10 (58.8%)	7 (41.2%)
Nodal status: N0+N1 (n=30)	21 (70%)	9 (30%)*	22 (73.3%)	8 (26.7%)
N2+N3 (n=15)	3 (20%)	12 (80%)*	9 (60%)	6 (40%)
Stage: I+II (n=17)	13 (76.5%)	4 (23.5%)*	13 (76.5%)	4 (23.5%)
III+IV (n=28)	11 (39.3%)	17 (60.7%)*	18 (64.3%)	10 (35.7%)

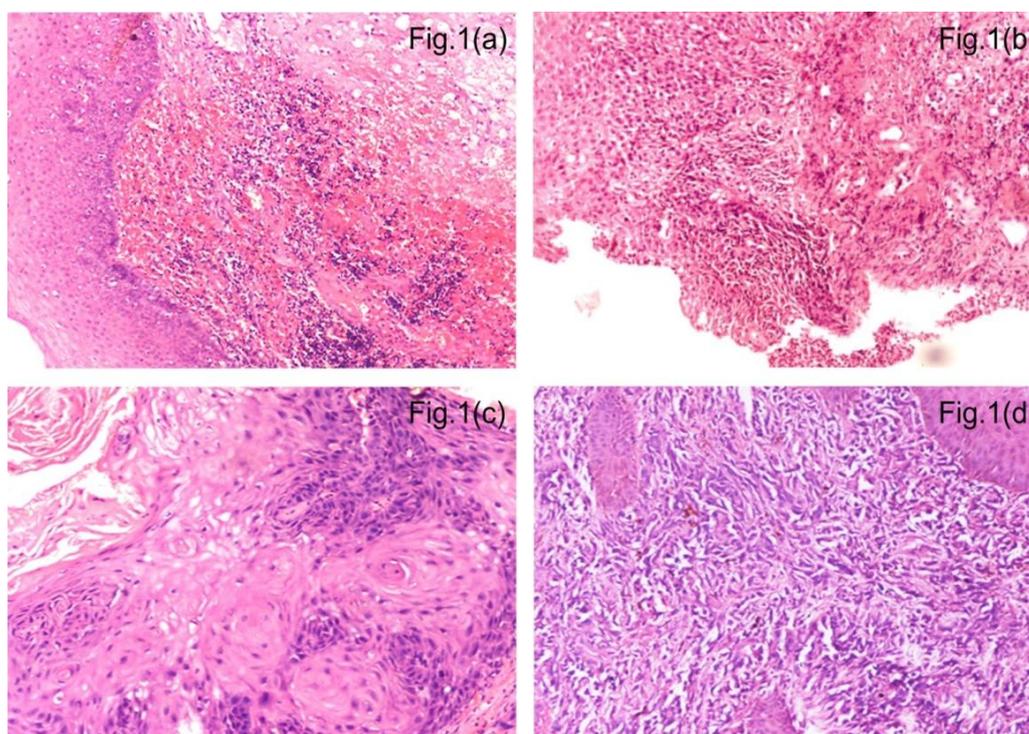


Figure 1: (a) Mild dysplasia (b) Severe dysplasia (c) Well differentiated squamous cell carcinoma (d) Poorly differentiated carcinoma (H&E 10X)

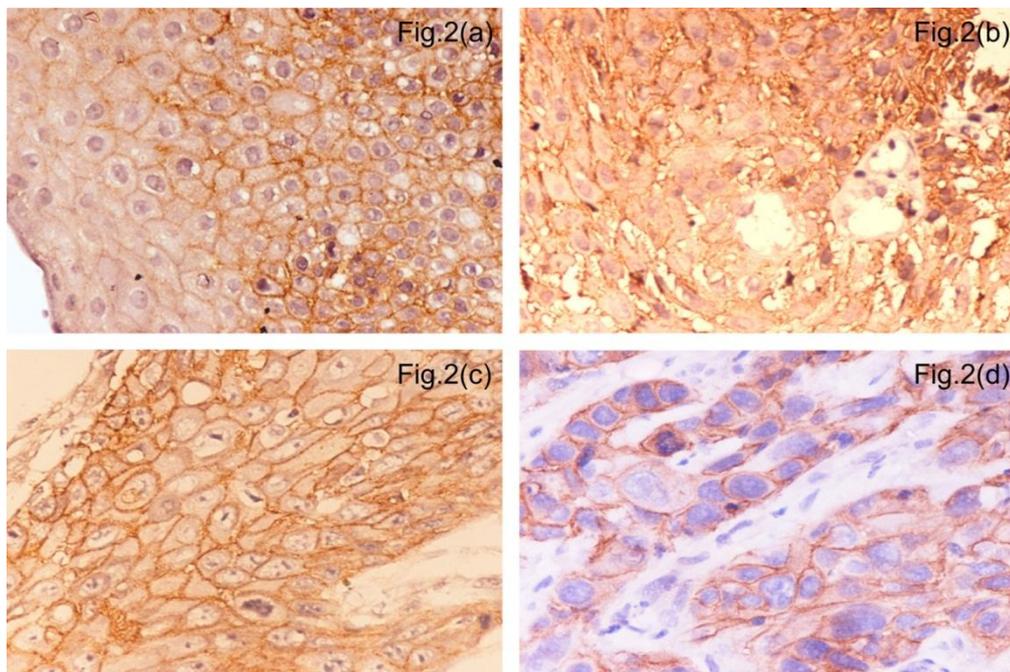


Figure 2: EGFR expression in (a) Mild dysplasia (b) Severe dysplasia (c) Well differentiated squamous cell carcinoma (d) Poorly differentiated carcinoma (IHC 40X)

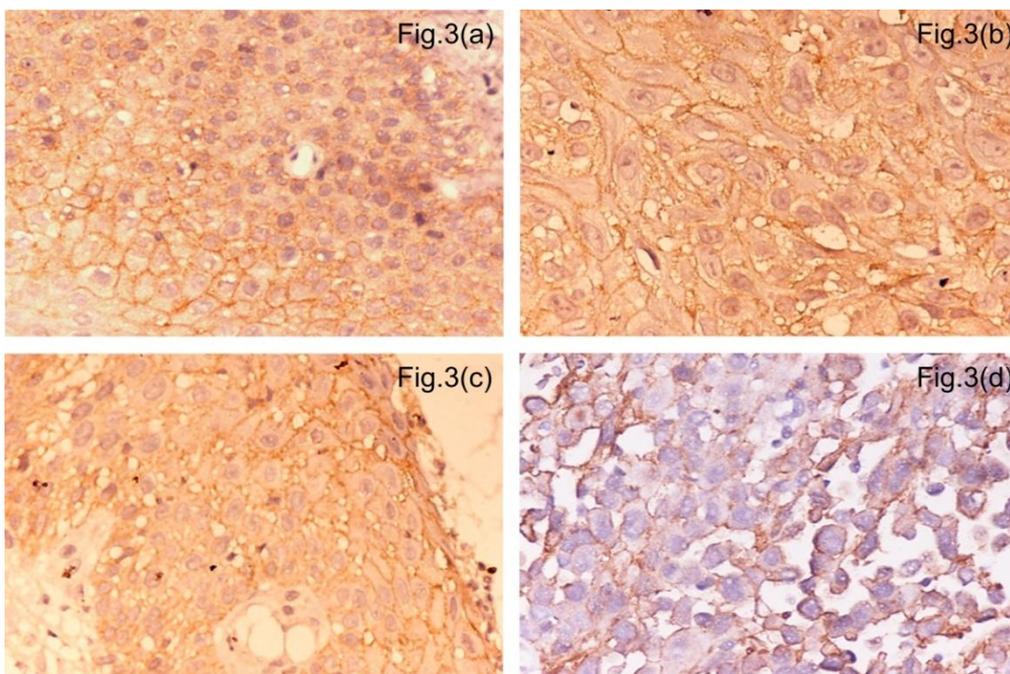


Figure 3: Her-2 neu expression in (a) Mild dysplasia (b) Severe dysplasia (c) Well differentiated squamous cell carcinoma (d) Poorly differentiated carcinoma (IHC 40X)

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