Immunohistochemical study of epidermal growth factor receptor, human epidermal growth factor receptor 2/neu, p53, and Ki67 in oral squamous cell carcinoma

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INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the most common malignant tumor occurring in the oral cavity.[1] In the majority of the cases, it is diagnosed at an advanced stage as a result of which bear poor prognosis. Worldwide, OSCC is the sixth most prevalent cancer, ranking eighth in developed countries and third in the developing world. It causes over 30% of all
cancers in India. It is the most common cancer in males and the third most common cancer in women in India.

The ErbB family consists of four closely related receptors which includes epidermal growth factor receptor (EGFR) (ErbB1/HER1), ErbB2 (HER2/neu), ErbB3 (HER3) and ErbB4 (HER4). These homologous family members are membrane-spanning tyrosine kinases that exist as inactive monomers. Upon ligand binding, the receptors homodimerize or heterodimerize with other ErbB protein family members triggering autophosphorylation of their intracellular tyrosine kinase domains and initiating a signaling cascade. The ErbB proteins are expressed in most epithelial cell layers and play a key role in cell differentiation during development.

EGFR signaling participates in the regulation of cell proliferation and differentiation during development. EGFR contributes to proliferation, invasion and metastasis in neoplastic cells. It has been documented to correlate in a variety of cancers especially OSCC with poor prognosis and resistance to radiotherapy.

HER2/neu (ErbB2, c-erbB2, or HER2) is a proto-oncogene located on human chromosome-17. It is overexpressed in several malignancies. However, studies on HER2/neu in OSCC are discordant and insufficient.

p53 is a tumor-suppressor gene located on the short arm (p) of chromosome 17. It encodes a protein TP53, whose mutation is one of the most common genetic aberration in oral carcinogenesis.

Proliferation marker such as Ki67 has been studied in OSCC. Ki-67 is a nuclear antigen expressed in dividing cells (S, G1, G2 and M phase of cell cycle) but nonexistent in resting cells (G0 phase).

The present study was conducted to evaluate the biomarkers such as EGFR, HER2/neu, p53 and Ki67 expression in OSCC cases and its correlation with other well-established clinicopathological parameters.

MATERIALS AND METHODS

This cross-sectional study was conducted in the department of pathology at a tertiary care hospital. Ethical clearance was obtained from Institutional Ethical Committee. Seventy cases of OSCC cases operated upon and diagnosed from 2015 to 2019 were included in the study. The available data for all the patients as regards with age, location of tumor, grade, stage and lymph node (LN) status were collected from the records of histopathology section of the department of pathology.

All the slides were evaluated to confirm or correct the previous histological diagnoses according to the revised criteria suggested by the World Health Organization by two senior histopathologists. Cases were divided into groups depending on LN metastasis, histological grade (low and high grade) and tumor volume (<8 cm³ and >8 cm³). Tumor volume was calculated as, longitudinal axis × width × depth.

The most suitable tissue block of OSCC cases was selected for IHC evaluation. A technique of manual tissue microarray was employed for the study of EGFR, HER2/neu, p53 and Ki67 in all cases with one tissue core taken from each selected OSCC block. Antigen retrieval was done using Citrate Buffer Antigen Retrieval Protocol. Pressure cooker was used as a heating source.

The primary antibodies used were EGFR (Clone EP 22, BioGenex), HER2/neu (Clone CB11, Novacastra), p53 (clone DO-7, Dako) and Ki-67 (Clone MIB-1, Dako). Negative controls (without adding primary antibody) were included in all batches. Appropriate positive controls were taken for the IHC stains as per the literature. Section from skin was used as positive control for EGFR expression. Section from breast carcinoma, which previously showed unequivocal strong immunoreactivity for HER2/neu, was used as positive control for HER2/neu. Section from prostate and skin was used as positive control for p53 and Ki67. Sections were examined under high power field to observe the immunoreactivity.

The staining for EGFR was considered positive when at least 10% or more of the tumor cells showed membrane expression of the marker with a weak to moderate to strong intensity of staining. The intensity of EGFR was scored on a scale from 1 to 3, where 1 = weak, 2 = moderate and 3 = strong homogenous or patchy staining.

The staining for HER2/neu was considered positive when tumor cells showed membrane expression of the marker which was scored on a scale from 0 to 3, where 0 = no staining, 1 = 10%–50% and 3 ≥50% stained tumor cells.

The staining for p53 was considered positive when at least 10% or more of the tumor cells showed nuclear expression of the marker. The p53 staining was scored on a scale from 1 to 3, where 1 = 10%–30%, 2 = 30%–50% and 3 ≥50% stained tumor cells.

Ki67 was evaluated as positive when >10% of tumor cells displayed moderate to strong nuclear staining.
The Primer of Biostatistics 7.0 program was used for the calculation of interrelationships between the analyzed EFGR, HER2/neu, p53 and Ki67 expression and clinicopathological variables by the Pearson's Chi-square test. Quantitative data were presented with the help of mean. Qualitative data were presented with the help of frequency and percentage table. The results were considered to be statistically significant when the P < 0.05 and highly statistically significant when P < 0.01.

RESULTS

The various clinicopathological features of OSCC are presented in Table 1. The age of patients ranged from 35 to 75 years, with a mean value of 52.86 years. The highest number of cases (24/70) was seen in the age group of 41–50 years (34.28%). Maximum number of OSCC cases in Stage IV (23/70, 32.9%) belonged to a higher grade of OSCC (moderately-differentiated SCC [MDSCC] + poorly-differentiated SCC [PDSCC]). It was found that the maximum number of cases in all stages, i.e., Stage I (6/70, 8.6%), Stage II (10/70, 14.3%), Stage III (6/70, 8.6%) and Stage IV (23/70, 32.9%), belonged to a high grade OSCC. However, the association between tumor stage and tumor grade in OSCC cases was not found to be statistically significant [χ² = 0.646, P = 1.000; Table 2]. The maximum number of cases showing tumor volume more than 8 cm³ belonged to high grade OSCC (24/70, 34.3%) (MDSCC + PDSCC). However, the association between tumor grade and volume was not found to be statistically significant [t = 1.427, P = 0.158; Table 3]. On comparison of the LN metastasis with grade of the tumor, maximum number of cases having LN metastasis (18/26, 69.2%) belonged to high grade SCC (MDSCC + PDSCC).χ² = 0.165, P = 0.685) [Table 4].

The EGFR, HER2/neu, p53 and Ki67 expression and its correlation with various clinicopathological parameters of OSCC cases is shown in Tables 5-8, respectively [Figure 1].

EGFR, HER2/neu, p53 and ki67 positivity were seen in 65/70 (92.9%), 32/70 (45.7%), 30/70 (42.9%) and 55/70 (78.6%) cases of OSCC. Maximum cases expressing EGFR, HER2/neu, p53 and Ki67 belonged to >50 year of age group, males and gingivobuccal sulcus as the site of the tumor. IHC analyses among the risk factor groups showed that maximum cases having tobacco usage expressed EGFR (20/70, 28.6%) and Ki67 (16/70, 22.9%) and showed negative HER2/neu expression (18/70, 25.71%) although statistically insignificant. Maximum cases expressing p53 (8/70, 11.4%) statistically significantly belonged to the group of cases showing tobacco usage.

EGFR expression increased as the grade of the tumor increased (χ² = 0.077, P = 0.782). The association between percentage of EGFR expression in tumor cells and the grade of tumor was statistically significant [t = −2.074, P < 0.05; Table 9]. EGFR expression significantly increased as the stage of the tumor increased (χ² = 36.152, P < 0.05).

Maximum number of cases showing LN metastasis expressed
EGFR ($\chi^2 = 1.014, P = 1.000$). Maximum p53 positive cases showed significant EGFR immunoeexpression ($\chi^2 = 4.877, P < 0.05$). Maximum Ki67 positive cases showed EGFR immunoeexpression ($\chi^2 = 0.235, P = 0.628$).

Maximum number of cases with HER2/neu immunoeexpression belonged to high grade OSCC (MDSCC + PDSCC = 25/70, 35.71%) ($\chi^2 = 3.87, P < 0.05$). The association between percentage of HER2/neu expression in tumor cells and the grade of tumor was statistically significant [$r = -2.170, P < 0.05$; Table 9]. HER2/neu showed significant positivity in the maximum number of OSCC cases of Stage III + IV (22/45, 41.89%) than those in Stage I + II (10/25, 40%) ($\chi^2 = 18.652, P < 0.05$). Majority of the oral SCC cases with LN metastasis showed HER2/neu immunoeexpression (13/26, 50.00%) ($\chi^2 = 5.443, P = 0.188$).

Maximum p53 positive cases (21/70, 30.00%) showed significant HER2/neu immunoeexpression ($\chi^2 = 1.153, P < 0.05$). Amongst HER2/neu positive cases, maximum cases of OSCC showed Ki67 immunoeexpression (26/32, 81.25%) ($\chi^2 = 0.044, P = 0.835$).

p53 immunoeexpression was seen in maximum number of high grade OSCC cases (29/70, 41.43%) ($\chi^2 = 3.596, P = 0.058$). The association between percentage of p53 expression in tumor cells and the grade of tumor was statistically significant [$r = -2.217, P < 0.05$; Table 9]. p53 showed positivity in maximum number of OSCC cases of Stage III + IV (26/45, 57.78%) than those in Stage I + II (14/25, 56.00%) ($\chi^2 = 2.42, P = 1.000$). Maximum cases showing LN metastasis expressed p53 (16/26, 61.54%) ($\chi^2 = 1.354, P = 0.980$).

| Stage of tumor | Tumor grade | Total, n (%) |
|----------------|-------------|--------------|
|                | Low (WDSCC), n (%) | High (MDSCC + PDSCC), n (%) |
| Stage I        | 2 (2.85) | 6 (8.57) | 8 (11.43) |
| Stage II       | 7 (10.00) | 10 (14.28) | 17 (24.28) |
| Stage III      | 3 (4.28) | 6 (8.57) | 9 (12.86) |
| Stage IV       | 13 (18.57) | 23 (32.86) | 36 (51.43) |

WDSCC: Well differentiated squamous cell carcinoma, MDSCC: Moderately differentiated squamous cell carcinoma, PDSCC: Poorly differentiated squamous cell carcinoma.

| Tumor volume (cm$^3$) | Tumor grade | Total, n (%) |
|-----------------------|-------------|--------------|
|                       | Low (WDSCC), n (%) | High (MDSCC + PDSCC), n (%) |
| <8                    | 10 (14.28) | 21 (30.00) | 31 (44.28) |
| >8                    | 15 (21.43) | 24 (34.28) | 39 (55.71) |

WDSCC: Well differentiated squamous cell carcinoma, MDSCC: Moderately differentiated squamous cell carcinoma, PDSCC: Poorly differentiated squamous cell carcinoma.

| Lymph node metastasis | Tumor grade | Total, n (%) |
|-----------------------|-------------|--------------|
|                       | Low (WDSCC), n (%) | High (MDSCC + PDSCC), n (%) |
| Present               | 18 (25.71) | 8 (11.43) | 26 (37.14) |
| Absent                | 27 (38.57) | 17 (24.28) | 44 (62.86) |

WDSCC: Well differentiated squamous cell carcinoma, MDSCC: Moderately differentiated squamous cell carcinoma, PDSCC: Poorly differentiated squamous cell carcinoma.
Ki67 immunoexpression was seen significantly in the maximum number of high grade (41/70, 58.57%) than low grade OSCC cases (14/70, 20.00%) ($\chi^2 = 9.775, P < 0.01$). The association between percentage of Ki67 expression in tumor cells and the grade of tumor was statistically significant ($t = −2.051, P < 0.05$; Table 9). In the present study, Ki67 immunoexpression was relatively higher in Stage (III + IV) cases of OSCC (36/45, 80.0%) as compared to those in Stage (I + II) (19/25, 76.00%) OSCC cases ($\chi^2 = 1.954, P = 0.796$). It was found that maximum number of cases with the presence of LN metastasis (22/26, 84.61%) showed Ki67 immunoexpression ($\chi^2 = 0.417, P = 0.518$).

### DISCUSSION

EGFR expression was seen in 92.9% of OSCC cases in this study. Most studies have reported EGFR expression in the range of 40% to 80%.[13] A study by Singla et al. reports 97.5% cases of OSCC expressing EGFR.[7] Our patients had tumors with higher grade and stage, which could account for the higher percentage of cases exhibiting EGFR expression. Ours is a charitable institute and caters to the rural population. Due to low socioeconomic status of these patients, OSCC cases are diagnosed in advanced stages.

| Clinical parameters | Total number of cases (n) | EGFR expression (Positive (65/70; 92.9%), n (%) | Negative (5/70; 7.1%), n (%) | $\chi^2$ | $P$ |
|---------------------|--------------------------|-----------------------------------------------|----------------------------|--------|-----|
| Age (years)         |                          |                                              |                            |        |     |
| <50                 | 30                       | 3 (2%)                                       | 22 (88.0%)                 | 6.648  | 0.119|
| >50                 | 40                       | 11 (27.5%)                                   | 26 (65.0%)                 |        |     |
| Sex                 |                          |                                              |                            |        |     |
| Male                | 47                       | 3 (6.4%)                                     | 31 (66.0%)                 | 6.079  | 0.013|
| Female              | 23                       | 1 (4.3%)                                     | 17 (73.9%)                 |        |     |
| Risk factors/habits |                          |                                              |                            |        |     |
| Alcohol             | 5                        | 1 (20.0%)                                    | 3 (60.0%)                  | 3.914  | 0.046|
| Tobacco             | 22                       | 1 (4.5%)                                     | 13 (59.1%)                 |        |     |
| Smoking             | 8                        | 0 (0%)                                        | 6 (75.0%)                  |        |     |
| Alcohol + tobacco   | 7                        | 0 (0%)                                        | 5 (71.4%)                  |        |     |
| Alcohol + smoking   | 9                        | 1 (11.1%)                                    | 5 (55.6%)                  |        |     |
| Tobacco + smoking   | 1                        | 0 (0%)                                        | 1 (100.0%)                 |        |     |
| Betal quid          | 4                        | 0 (0%)                                        | 4 (100.0%)                 |        |     |
| Tobacco + betal quid| 3                        | 0 (0%)                                        | 2 (66.7%)                  |        |     |
| No addictions       | 11                       | 1 (9.1%)                                     | 9 (81.8%)                  |        |     |
| Site of the tumor   |                          |                                              |                            |        |     |
| GBS                 | 36                       | 10 (27.8%)                                   | 22 (58.3%)                 | 43.132 | 0    |
| BM                  | 13                       | 2 (15.4%)                                    | 9 (76.9%)                  |        |     |
| Tongue              | 16                       | 1 (6.25)                                     | 13 (81.25%)                |        |     |
| Lip                 | 1                        | 1 (100.0%)                                   | 0 (0%)                     |        |     |
| FOM                 | 1                        | 0 (0%)                                        | 1 (100.0%)                 |        |     |
| HP                  | 1                        | 0 (0%)                                        | 1 (100.0%)                 |        |     |
| RMT                 | 2                        | 2 (100.0%)                                   | 0 (0%)                     |        |     |
| Tumor grade         |                          |                                              |                            |        |     |
| WDSCC               | 25 (35.7)                | 0 (0%)                                        | 18 (72%)                   | 0.077  | 0.782|
| MDSCC               | 26 (37.1)                | 3 (11.5)                                     | 18 (69.3%)                 |        |     |
| PDSCCC              | 19 (27.2)                | 1 (5.3)                                       | 12 (63.2%)                 |        |     |
| Tumor stage         |                          |                                              |                            |        |     |
| I                   | 8 (11.4)                 | 1 (12.5%)                                    | 5 (62.5%)                  | 36.152 | <0.05|
| II                  | 17 (24.3)                | 1 (6.0%)                                     | 13 (76.5%)                 |        |     |
| III                 | 9 (12.9)                 | 1 (11.1%)                                    | 5 (55.6%)                  |        |     |
| IV                  | 36 (51.4)                | 12 (33.3%)                                   | 25 (69.4%)                 |        |     |
| Lymph node metastasis|                        |                                              |                            |        |     |
| Present             | 26 (37.1)                | 1 (4.0%)                                     | 19 (73.1%)                 | 1.014  | 1    |
| Absent              | 44 (62.9)                | 3 (6.8%)                                     | 29 (65.9%)                 |        |     |
| p53                 |                          |                                              |                            |        |     |
| Positive            | 40 (57.1)                | 0 (0%)                                        | 40 (97.1%)                 | 4.877  | <0.05|
| Negative            | 25 (35.7)                | 5 (20.0%)                                    | 20 (80.0%)                 |        |     |
| Ki67                 |                          |                                              |                            |        |     |
| Positive            | 52 (74.3)                | 3 (4.3%)                                     | 49 (61.4%)                 | 0.235  | 0.628|
| Negative            | 13 (18.6)                | 2 (29.4%)                                    | 11 (1.4%)                  |        |     |

EGFR: Epidermal growth factor receptor, GBS: Gingivobuccal sulcus, BM: Buccal mucosa, FOM: Floor of mouth, HP: Hard palate, RMT: Retromolar trigone WDSCC: Well differentiated squamous cell carcinoma, MDSCC: Moderately differentiated squamous cell carcinoma, PDSCC: Poorly differentiated squamous cell carcinoma.
It has documented that high EGFR expression suggests an uncontrolled growth mediated by EGFR overexpression. However, a study has shown significant correlation between gene amplification and micro-RNA expression, but no such correlation was noted between EGFR protein overexpression and micro-RNA expression. This goes to suggest EGFR expression is not regulated transcriptionally and some other mechanisms comes to play for observed EGFR overexpression in OSCC cases.

In this study, majority of the OSCC cases exhibited marked (48/70, 68.57%) followed by moderate (13/70, 18.57%) and weak (4/70, 5.71%) EGFR expression. This is according to the scoring system adopted by Young et al. and followed as optimum methodology by extensive search by Verma et al.[13]

There was a significant positive association between percentage of tumor cells expressing EGFR and grade of tumor in this study. There are conflicting reports of preferential expression of EGFR in either well or poorly differentiated tumors documented in the literature. Our study was similar to studies done by Singla et al. and Shiraki et al.[7,14] However, in contrary to this, Bernardes et al. and Verma et al. documented in their study that majority of low grade OSCC cases showed statistically insignificant EGFR immunoeexpression.[12,13] Verma et al. in their study documented similar EGFR expression in all grades of OSCC.
In the present study, the maximum number of low (well-differentiated squamous cell carcinoma) (18/25, 72%) and high (MDSCC + PDSCC) (30/45, 66.66%) grade OSCC cases showed Grade 3+ EGFR positivity. Similar findings were also noted in a study done by Singla et al. in which it was documented that EGFR overexpression can be significantly correlated with poor tumor differentiation. In this study, EGFR immunoexpression increased significantly as the stage of cancer increased from Stage I to Stage IV as documented in the literature, although not statistically significant. Bernardes et al. in contrary showed that EGFR immunoexpression was more in low grade OSCC. Furthermore, the maximum number of cases in the present study in each stage of oral SCC showed Grade 3+ EGFR positivity. This may be attributed to the fact that the maximum number of cases in each stage belonged to a high grade OSCC, which was similar to the finding of the study done by Verma et al., where the maximum number of OSCC cases showed Grade 2 + followed by Grade 3 + EGFR positivity.

In this study, EGFR immunoexpression was seen insignificantly in maximum number of cases showing LN metastasis as documented in literature. However, in a study by Verma J et al., majority of the OSCC cases with the absence of LN metastasis showed EGFR immunoexpression. In this study, it was found that in LN positive cases, maximum number of cases showed Grade 3+ EGFR positivity. In the study conducted by Verma J et al., majority of the OSCC cases with LN metastasis showed Grade 2+ followed by Grade 3+ EGFR positivity.

Maximum p53-positive cases showed statistically significant EGFR immunoexpression in this study. Only few studies were found showing the correlation of EGFR and p53

Table 7: p53 expression and clinicopathological parameters of oral squamous cell carcinoma

| Clinical parameters | Total number of cases (n) | p53 expression | \( \chi^2 \) | P |
|---------------------|--------------------------|----------------|----------|----|
| Age (years)         |                          |                |          |    |
| <50                 | 30                       | 9 5 4 18 (25.7) | 12 (17.1) | 4.547 0.278 |
| >50                 | 40                       | 9 12 1 22 (31.4) | 18 (25.7) |
| Sex                 |                          |                |          |    |
| Male                | 47                       | 15 11 1 27 (38.6) | 20 (28.6) | 7.225 0.085 |
| Female              | 23                       | 3 6 4 13 (18.6) | 10 (14.3) |
| Risk factors/habits |                          |                |          |    |
| Alcohol             | 5                        | 0 0 0 5 (7.1) | 0 | 17.646 <0.05 |
| Tobacco             | 22                       | 0 5 3 8 (11.4) | 14 (20) |
| Smoking             | 8                        | 1 4 1 6 (8.6) | 2 (2.9) |
| Alcohol + tobacco   | 7                        | 0 2 0 2 (2.9) | 5 (7.1) |
| Alcohol + smoking   | 9                        | 2 3 1 6 (8.6) | 3 (4.3) |
| Tobacco + smoking   | 1                        | 0 1 0 1 (1.4) | 0 |
| Betal quid          | 4                        | 0 2 2 4 (5.7) | 0 |
| Tobacco + betal quid| 3                        | 0 2 1 3 (4.3) | 0 |
| No addictions       | 11                       | 0 6 1 7 (10) | 4 (5.7) |
| Site of the tumor   |                          |                |          |    |
| GBS                 | 36                       | 8 10 2 20 (28.6) | 16 (22.9) | 15.502 0.627 |
| BM                  | 13                       | 5 1 0 6 (8.6) | 7 (10.0) |
| Tongue              | 16                       | 5 4 3 12 (17.1) | 4 (5.7) |
| Lip                 | 1                        | 0 0 0 1 (100) | |
| FOM                 | 1                        | 0 1 0 1 (100) | |
| HP                  | 1                        | 0 0 0 1 (100) | 0 |
| RMT                 | 2                        | 0 1 0 1 (50) | 1 (50) |
| Tumor grade         |                          |                |          |    |
| WDSCC               | 25 (35.7)                | 4 (36.4) 7 (63.6) | 0 11 (44.0) | 14 (56.0) | 3.596 0.058 |
| MDSCC               | 26 (37.1)                | 7 (50.0) 6 (42.9) | 1 (7.1) | 14 (53.8) | 12 (46.2) |
| PDSCCC              | 19 (27.2)                | 7 (46.6) 4 (26.7) | 4 (26.7) | 15 (78.9) | 4 (21.1) |
| Tumor stage         |                          |                |          |    |
| I                   | 8 (11.4)                 | 2 (50.0) 2 (50.0) | 0 4 (50.0) | 4 (50.0) | 2.42 1 |
| II                  | 17 (24.3)                | 4 (40) 4 (40) | 2 (20) | 10 (58.8) | 7 (41.2) |
| III                 | 9 (12.9)                 | 2 (40) 3 (60) | 0 5 (55.6) | 4 (44.4) |
| IV                  | 36 (51.4)                | 10 (47.6) 8 (38.1) | 3 (14.3) | 21 (58.3) | 15 (41.7) |
| Lymph node metastasis|                        |                |          |    |
| Present             | 26 (37.1)                | 7 (43.7) 6 (37.5) | 3 (18.8) | 16 (61.5) | 10 (38.5) | 1.354 0.98 |
| Absent              | 44 (62.9)                | 11 (45.8) 11 (45.8) | 2 (8.4) | 24 (54.5) | 20 (45.5) |

GBS: Gingivobuccal sulcus, BM: Buccal mucosa, FOM: Floor of mouth, HP: Hard palate, RMT: Retromolar trigone WDSCC: Well differentiated squamous cell carcinoma, MDSCC: Moderately differentiated squamous cell carcinoma, PDSCC: Poorly differentiated squamous cell carcinoma
immunoexpression such as Singla et al. and Shiraki et al.[7,14] One author concludes that co-expression of p53 and EGFR is associated with an invasive growth pattern and worse survival. OSCC cases simultaneously expressing p53 and EGFR had a significantly worse prognosis than groups with no or single marker expression.[14] In contrary, Parise et al. concluded that p53 positivity and EGFR negativity in OSCC may be a prognostic factor for survival.[15] Both p53 and EGFR are interlinked to each other at a molecular level and may augment each other in cases of carcinogenesis.[1] Mutant p53 binds to promote a sustained EGF-induced extracellular signal regulated kinase 1/2 activation, thereby facilitating cell proliferation and tumorigenesis.[16] Maximum Ki67-positive cases showed insignificant EGFR immunoexpression in this study similar to a study which states that EGFR overexpression was seen in most Ki67 positive OSCC cases.[17]

**Table 8: Ki67 expression and clinicopathological parameters of oral squamous cell carcinoma**

| Clinical parameters | Total number of cases | Ki67                          | χ²  | P      |
|--------------------|-----------------------|-------------------------------|-----|--------|
| Positive, n (%)    | Negative, n (%)       |                               |     |        |
| Age (years)        |                       |                               |     |        |
| <50                | 30                    | 24 (34.3)                     | 6 (8.6) | 0.002 | 0.966 |
| >50                | 40                    | 31 (44.3)                     | 9 (12.6)| 6.82  | 0.009 |
| Sex                |                       |                               |     |        |
| Male               | 47                    | 36 (51.4)                     | 11 (15.7)| 0.071 | 0.79  |
| Female             | 23                    | 19 (27.1)                     | 4 (5.7)|        |       |
| Risk factors/habits|                       |                               |     |        |
| Alcohol            | 5                     | 3 (4.3)                       | 2 (2.9)| 7.764 | 0.457 |
| Tobacco            | 22                    | 16 (22.9)                     | 6 (8.6)|        |       |
| Smoking            | 8                     | 7 (10)                        | 1 (1.4)|        |       |
| Alcohol + tobacco  | 7                     | 6 (8.6)                       | 1 (1.4)|        |       |
| Alcohol + smoking  | 9                     | 4 (5.7)                       | 5 (7.1)|        |       |
| Tobacco + smoking  | 1                     | 0                             | 1 (1.4)|        |       |
| Betal quid         | 4                     | 2 (2.9)                       | 2 (2.9)|        |       |
| Tobacco + betal quid| 3                    | 2 (2.9)                       | 1 (1.4)|        |       |
| No addictions      | 11                    | 7 (10)                        | 4 (5.7)|        |       |
| Site of the tumor  |                       |                               |     |        |
| GBS                | 36                    | 28 (40)                       | 8 (11.4)| 2.119 | 0.908 |
| BM                 | 13                    | 9 (12.9)                      | 4 (5.7)|        |       |
| Tongue             | 16                    | 13 (18.6)                     | 3 (4.3)|        |       |
| Lip                | 1                     | 1 (100)                       | 0    |        |       |
| FOM                | 1                     | 1 (100)                       | 0    |        |       |
| HP                 | 1                     | 1 (100)                       | 0    |        |       |
| RMT                | 2                     | 2 (100)                       | 0    |        |       |
| Tumor grade        |                       |                               |     |        |
| WDSCC              | 25                    | 14 (20)                       | 11 (15.7)| 9.775 | <0.001|
| MDSCC              | 26                    | 22 (31.4)                     | 4 (5.7)|        |       |
| PDSCC              | 19                    | 19 (27.1)                     | 0    |        |       |
| Tumor stage        |                       |                               |     |        |
| I                  | 8                     | 5 (62.5)                      | 3 (37.5)| 1.954 | 0.796 |
| II                 | 17                    | 14 (82.4)                     | 3 (17.6)|        |       |
| III                | 9                     | 8 (88.9)                      | 1 (11.1)|        |       |
| IV                 | 36                    | 28 (77.8)                     | 8 (22.2)|        |       |
| Lymph node metastasis|                     |                               |     |        |
| Present            | 26                    | 22 (84.6)                     | 4 (15.4)| 0.417 | 0.518 |
| Absent             | 44                    | 33 (75.0)                     | 11 (25.0)|        |       |

GBS: Gingivobuccal sulcus, BM: Buccal mucosa, FOM: Floor of mouth, HP: Hard palate, RMT: Retromolar trigone WDSCC: Well differentiated squamous cell carcinoma, MDSCC: Moderately differentiated squamous cell carcinoma, PDSCC: Poorly differentiated squamous cell carcinoma

**Table 9: Association between tumor grade and biomarkers in oral squamous cell carcinoma cases**

| Biomarker | Tumor grade | Number of cases | Mean | SD  | SEM | T   | P       |
|-----------|-------------|-----------------|------|-----|-----|-----|--------|
| EGFR      | Low grade   | 25              | 38.2 | 16.44| 3.28| −2.074| <0.05  |
|           | High grade  | 45              | 51.77| 30.26| 4.51|     |        |
| HER2/neu  | Low grade   | 25              | 5.8  | 17.44| 3.48| −2.17 | <0.05  |
|           | High grade  | 45              | 15.24| 17.44| 2.6 |     |        |
| p53       | Low grade   | 25              | 10.8 | 21.15| 4.2 | −2.217| <0.05  |
|           | High grade  | 45              | 23.44| 23.74| 3.5 |     |        |
| Ki67      | Low grade   | 25              | 14.64| 12.3 | 2.46| −2.051| <0.05  |
|           | High grade  | 45              | 22.97| 18.11| 2.77|     |        |

SD: Standard deviation, SEM: Standard error of mean, EGFR: Epidermal growth factor receptor, HER2/neu: Human epidermal growth factor receptor

HER2/neu was expressed in 45.7% of OSCC cases. Wide variation in HER2/neu variation is observed in the literature. This disparity in results is related to the clinicopathological parameters of OSCC cases.[18] Most studies fail to mention the scores of intensities of HER2/neu expression which we have specifically mentioned in this study.[19]
Maximum number of cases with HER2/neu immunoexpression belonged to high grade OSCC as documented in the literature. The association between percentage of HER2/neu expression in tumor cells and the grade of tumor was statistically significant. In the present study, distinct membranous expression of HER2/neu was considered as positive finding, whereas some studies considered both cytoplasmic and membranous expression as positive HER2/neu expression. However, it is argued that cytoplasmic staining may be a technical artifact due to cross-reactive antibodies possibly with keratin or during antigen retrieval. HER2/neu showed significant positivity in the maximum number of OSCC cases of Stage III + IV than those in Stage I + II as documented in literature by Vats et al. and Cavalot et al.

Majority of the oral SCC cases with LN metastasis showed insignificant HER2/neu immunoexpression as documented in literature. Maximum p53 positive cases showed HER2/neu immunoexpression though statistically insignificant. In contrary, Singla et al. stated that the expression of HER2/neu was negative in all OSCC cases. Patise et al. concluded the absence of correlation between HER2/neu and p53 immunoexpression in their study was due to loss of mucosal HER2/neu expression in squamous cell carcinogenesis. Maximum Ki67 positive cases showed insignificant HER2/neu immunoexpression in this study similar to that documented in the literature.

p53 immunoexpression was seen in the maximum number of high grade OSCC cases similar to Singla et al. The association between percentage of p53 expression in tumor cells and the grade of tumor was statistically significant. Monteiro et al. also reported increased expression of p53 in moderately and poorly differentiated carcinomas as compared to well-differentiated carcinomas. p53 immunoexpression was found in the maximum number of cases with LN metastasis as documented in the literature. p53 encodes a protein TP53, whose mutation is one of the most common events in oral carcinogenesis. The gene mutation produces an accumulation of p53 protein, which can be detected by IHC methods and its overexpression has been associated with the poor survival of patients with OSCCs. Normal tissue expresses wild-type p53 which has a short half-life and most of it is not detected IHC. By contrast, mutations of p53 result in a greatly extended protein half-life, thus permitting IHC detection. Unlike the proteins of nontransformed cells, the mutant protein is likely to form complexes leading to the acquisition of a stable conformation than the wild-type protein. Thus, it is suggested that the overexpression of p53 is a common event in the multistep carcinogenesis in OSCCs.

Ki67 immunoexpression was seen significantly in high grade than low grade OSCC cases as documented by most studies. The association between percentage of Ki67 expression in tumor cells and the grade of tumor was statistically significant. In the study of Singh S et al., comparison of Ki67 expression between the three grades of OSCC cases, showed that poorly differentiated group had the highest value and well differentiated had the least value. This difference was statistically significant. Ki67 immunoexpression was relatively higher in Stage (III + IV) cases of OSCC as compared to those in Stage (I + II) OSCC cases in this study as documented by Bhayekar et al. It was found that maximum number of cases with the presence of LN metastasis showed Ki67 immunoexpression. One study noticed that a strong trend toward Ki-67 positive immunoexpression in tumors with neck metastasis. Thus, representing an independent prognostic factor in the survival of patients with OSCC cases.

IHC analyses among the risk factor groups showed that maximum cases having tobacco usage expressed EGFR and Ki67 and showed negative HER2/neu expression although statistically insignificantly. Maximum cases expressing p53 statistically significantly belonged to the group of cases showing tobacco usage. This is more or less similar to the findings documented in literature.

For a molecule to be an optimum candidate as a target for anticancer therapy, the protein must be overexpressed in cancerous as compared to normal tissues and this overexpressed protein should be associated with bad prognosis which proposes that the protein manipulation may result in alteration of the prognosis. In this study, both EGFR and HER2/neu have these characteristics. Recently, targeting of EGFR and HER2/neu as a molecular adjuvant therapy has been clinically tried in OSCC cases.

This study intends to document prognostic utility of EGFR and HER2/neu expression in OSCC cases in the Indian setting and contribute to the data pool which could aid in formulating individual tailored therapy that includes targeted therapy in oral SCC cases.

Limitations
TMA technique was used for EGFR, HER2/neu, p53 and Ki67. Whole sections were not used for their IHC evaluation. However, utmost care was taken to sample...
the most representative area from the original whole section blocks for TMA. HER2 was assessed only by IHC. Evaluation by FISH was not available, especially for the equivocal cases with HER2 expression 2+. Follow-up time for the patients was limited.

CONCLUSION
A statistically significant positive association was noted between EGFR expression and tumor grade, tumor stage and p53 immunoexpression in OSCC cases. Increased EGFR expression was noted insignificantly in OSCC cases with LN metastasis and Ki67-positive cases. Statistically significant positive association was noted between HER2/neu expression and tumor grade and stage of oral SCC cases. Increased HER2/neu expression was noted insignificantly in OSCC cases with LN metastasis, p53 and Ki67 positive OSCC cases. A statistically significant positive association was noted between percent of tumor cells expressing EGFR, HER2/neu, p53 and Ki67 and grade of OSCC. EGFR, HER2/neu, p53 and ki67 immunoexpression could be routinely incorporated into surgical pathology report as a prognostic marker which could help in better patient management. OSCC showing EGFR and HER2/neu immunoexpression may benefit from specific targeted therapy.

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Conflicts of interest
There are no conflicts of interest.

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