International Journal of Science and Research (IJSR)
ISSN: 2319-7064
SJIF (2022): 7.942

# HPV-p16 as Major Etiological Agent in Causation of Oral Squamous Cell Carcinoma

**Dr. Ankita Gupta[1], Dr. N. S. Mani[2]**

[1, 2]Department of Pathology, BharatiVidyapeeth Medical College, Pune, Maharashtra, India

**Abstract:** ***According to recent WHO 2020 statastics Oral cancers ranked as 13th most common cancer worldwide. The global incidence of cancers of the lip and oral cavity is estimated to be 3, 77, 713 new cases and 1, 77, 757 deaths in 2020. [1] Oral cancer is more common in men and in older people, more deadly in men compared to women and it varies strongly by socio-economic circumstances and it is 20 per 100000 population that makes 30% of overall cacers in India. [1]Multiple studies have been published stating tobacco and betel nut chewing, smoking, alcohol consumption as major etiological factor in causation of Oral squamous cell carcinoma (OSCC). This is multifactorial from which we focussed on our aim to establish Human Papilloma Virus (HPV)-p16 as one of the major etiological agent in the causation of OSCC. Hence this study includes three hierarchial molecular marker p16-a molecular marker for HPV, Epidermal Growth Factor Receptor (EGFR) as pathway activator, and Ki67 as cellular proliferation factor. [2] This observational study included 78 cases of both biopsies and resection specimens and studied various other parameters like age, gender, presentation of lesion, site distribution, histomorphological spectrum and complete TNM staging and stage grouping for 31 resection cases. All mentioned parameters are then correlated with the so said IHC markers-p16, EGFR and Ki67 and results were analyzed and concluded.***

**Keywords:** Oral squamous cell carcinoma, Immunohistochemistry, p16, HPV

## 1. Introduction

The Pathogenesis of OSCC is multifactorial in which exogenous agents like high risk HPV are implicated. HPV positive related oncogenes – oncoproteins, inactivation of tumor suppressor protein's, activation of cyclins, inhibition of apoptosis and continued cellular senescence. The corresponding oncogenes of GFR remain active beyond normal regulatory mechanism of which EGFR is prototype. Increased nuclear transcription marker is one of the hallmarks of cancers reflecting increased mitotic activity.

## 2. Material and Methods

This was an observational study that includes 74 cases of oral squamous cell carcinoma. Rest 04 cases were of hyperplasia and dysplasia. Both resection specimens and biopsies are included. The blocks from archieve was retrieved for retrospective cases. The tissue were processed for paraffin blocks. For freshly cut tissue appropriate sections were selected for Hematoxylin and eosin stain. Further the tissue sections were processed for specific immunohistochemistry using Peroxidase-Antiperoxidase method. The antibodies used were as follows:-

- p16 (MX-007)
- EGFR (EP22)
- Ki67 (SP6)

The collected data were coded and entered into a Microsoft Excel sheet. The data were analyzed using SPSS (Statistical Package for social sciences) version 260 software. The results were presented in a graphical and tabular format. P16 grading was carried out according to cytoplasmic and nuclear staining intensity and graded as 0 for Negative staining, 1+ for 1%-25% positivity, 2+ for 26%-50% positivity, 3+ for 51%-75% positivity and 4+ for 76%-100% positivity. EGFR scoring was assessed for the proportion of cells showing positive staining and was graded as 0 for negative staining, 1+ for weak staining, 2+ for moderate staining and 3+ for strong staining. The intensity was scored as 0-negative staining, 1 for 50% positive staining. The above two scores were added and were given final scores as 0 for no staining, 1-2 for weak expression, 3-4 for moderate expression and 5-6 for strong staining. Ki67 proliferation index was calculated as the number of positive cells divided by total number of cells multiplied by 100 in tumor hotspot areas. Then the mean labelling index and one standard deviation was calculated and recorded.

## 3. Results

**Table 1:** Age and Gender distribution. The mean age of all the patients was 54.32 ± 13.52 years, ranging between 22 to 85 years

| Age | Male | Female | n (%) |
|---|---|---|---|
| <=50 | 14 | 13 | 27 (41%) |
| >50 | 39 | 12 | 51 (59%) |
| Total | 53 (67.9%) | 25 (32.1%) | 78 (100.0%) |

**Table 2:** Site distribution. The commonest site of lesion was posterior GBS in 25 (32.0%) cases, followed by cheek mucosa in 23 (29.5%), anterior GBS in 21 (26.9%), retromolar area in 6 (7.6%) and tongue with 3 (3.8%) cases

| Site | No. of cases | percentage % |
|---|---|---|
| Cheek mucosa | 23 | 29.5 |
| Retromolar area | 06 | 7.6 |
| Ant GBS | 21 | 26.9 |
| Post GBS | 25 | 32.0 |
| Tongue | 03 | 3.8 |
| Total | 78 | 100.0 |

**Table 3:** Histomorphological distribution. There were only 3 cases (3.8%) of hyperplasia and 1 case (1.3%) of moderate dysplasia. All the remaining 74 cases were of squamous cell carcinomas (SCC). The histopathological classification showed 4 (5.2%) cases of acantholytic type SCC, 2 (2.5%) cases of verrucous type SCC and only 1 (1.3%) case of spindle cell type of SCC. The large majority i. e.67 cases of 78 (85.8%) cases of conventional squamous cell carcinomas

showed non keratinizing pattern of SCC. The histopathological grading of these 74 cases of SCC revealed that majority of the cases i. e.45 (57.6%) of the patients had moderately differentiated carcinoma, followed by well differentiated carcinoma in 28 cases (35.8%), and 1 case (1.3%) showed poorly differentiated carcinoma.

| Histomorphological diagnosis | No. of cases | percentage % |
|---|---|---|
| Hyperplasia | 03 | 3.8% |
| Dysplasia | 01 | 1.3% |
| SCC-conventional | 67 | 85.8% |
| Acantholytic SCC | 04 | 5.2% |
| Verrucous SCC | 02 | 2.5% |
| Spindled SCC | 01 | 1.3% |
| Total | 78 | 100.0% |

**Table 4:** The T stage for TNM staging according to CAP protocol is shown here. The tumor size along with depth of invasion was taken into account for final T staging when T2 (size >2 to<=4 cm with DOI <=10 mm or <= 2cm with DOI > 5mm) showed majority of cases i. e.09 (29.1%) cases followed by T4a (Size >4cm with DOI >10mm or invasion into adjacent structures) i. e.08 (25.8%) followed by, T1 (size<=2 cm with DOI <=5 mm) showed 07 (22.5%) cases and T3 (size >2 to <=4 cm with DOI >10 mm or > 4cm with DOI <=10mm) showed 05 (16.2%) cases and T4b showed least number of cases i. e.02 (6.4%).

| Tumorsize (cm) and DOI (mm) | No. of cases | Percentage % |
|---|---|---|
| T1= <=2/<=5 | 07 | 22.5 |
| T2= >2 to<=4/ <=10 or <=2/ >5 | 09 | 29.1 |
| T3= >2 to <=4/ >10 or >4/ <=10 | 05 | 16.2 |
| T4a = >4/ >10 or invasion into adjacent structures | 08 | 25.8 |
| T4b= very advanced local disease | 02 | 6.4 |
| Total | 31 | 100.0 |

**Table 5:** Nodal status. No regional lymph node metastasis (N0) was reported in the majority of cases (21 (67.7%)).4 (12.9%) cases showed nodal metastasis in 1-3 regional ipsilateral nodes (N1) and 4-6 regional nodes (N2) each. Only 2 (2.7%) cases showed involvement of more than 7 regional nodes (N3)

| Nodal Status | No. of Cases | Percentage % |
|---|---|---|
| N0 | 21 | 67.7 |
| N1 | 04 | 12.9 |
| N2 | 04 | 12.9 |
| N3 | 02 | 6.5 |
| Total | 31 | 100 |

**Table 6:** Stage grouping table. The group staging for 31 resection cases was carried out according to TNM classification and it showed, 03 (9.7%) cases were of stage I, 09 (29.0%) cases of stage II, 04 (12.9%) cases of stage III followed by majority of cases i. e.11 (35.5%) cases of stage IVA and 04 (12.9%) cases of stage IVB

| Stage Grouping | No. of cases | Percentage % |
|---|---|---|
| I (T1N0M0) | 03 | 9.7 |
| II (T2N0M0) | 09 | 29.0 |
| III (T1-T2-T3, N1, M0) | 04 | 12.9 |
| IVA (T4a, N0, M0 or N1, M0)<br>(T1-T4a, N2, M0) | 04<br>07 | 35.5 |
| IVB (T4b, any N, M0)<br>(Any T, N3, M0) | 02<br>02 | 12.9 |
| TOTAL | 31 | 100.0 |

**Table 7:** p16 staining intensity.26 out of 78 (33.3%) cases showed positivity for p16, while 52 out of 78 cases (66.7%) showed negative staining.14 cases out of 31 cases of resections (45.1%) were positive and 12 out of 47 (25.5%) cases of biopsies were positive. The distribution of cases as per intensity of expression was 1+ in 18 (69.2%) cases, and 2+ and 3+ in 4 (15.4%) cases each. Majority of the cases were negative for p16 stain, and none of the cases showed p16 expression of 4+.

| Staining intensity | No. of cases | Percentage % |
|---|---|---|
| 1+ | 18 | 69.2 |
| 2+ | 04 | 15.4 |
| 3+ | 04 | 15.4 |
| Total | 26 | 100 |

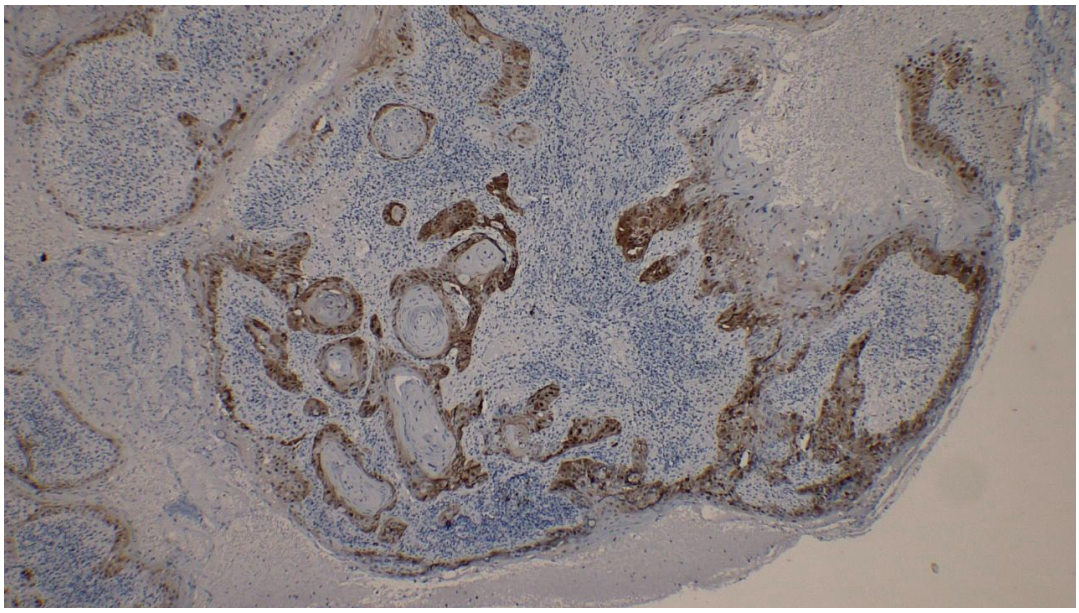

**Image 1:** p16 stain showing 3+ expression.

**Table 8:** Site correlation with p16 staining. p16 expression was correlated with the sites of the lesions as given in Table 17. Maximum expression of p16 was seen in cases from the posterior GBS in 15 (57.6%) cases followed by 7 (26.9%) cases in cheek mucosa, 2 (7.6%) cases of retromolar area, and 1 (3.8%) case each at anterior GBS and tongue

| Site | No. of cases | percentage % |
|---|---|---|
| Cheek mucosa | 07 | 26.9 |
| Retromolar area | 02 | 7.6 |
| Anterior GBS | 01 | 3.8 |
| Posterior GBS | 15 | 57.6 |
| Tongue | 01 | 3.8 |
| Total | 26 | 100 |

**Table 9:** p16 expression was correlated with histology and differentiation as given. Of which 2 out of 3 (66.6%) cases of hyperplasia and the only (100.0%) case of dysplasia were p16 positive.15 cases (33.3%) of moderately differentiated carcinomas showed p16 expression, followed by 8 cases (28.5%) of well differentiated carcinoma. The only poorly differentiated carcinoma was p16 negative

| Histology | P16 positive Cases | P16 negative cases | Frequency % |
|---|---|---|---|
| Hyperplasia | 02 | 01 | 66.6 |
| Dysplasia | 01 | 00 | 100.0 |
| Well differentiated Carcinoma | 08 | 20 | 28.5 |
| Moderately differentiated Carcinoma | 15 | 30 | 33.3 |
| Poorly differentiated Carcinoma | 00 | 01 | 00.0 |
| Total | 26 | 52 | 100.0 |

**Table 10:** Correlation of differentiation of carcinomas with intensity of p16 expression. The intensities of p16 expression was 2+ in one case of dysplasia and 3+ in both the cases of hyperplasia.18 out of 23 cases of oral SCC showed 1+ intensities of expression.12 out of 15 (80.0%) cases were moderately differentiated carcinomas, followed by 06 out of 08 (75.0%) cases of well differentiated carcinoma and poorly differentiated carcinoma showed no staining

| Histology | 1+ positive p16 cases | 2+ positive p16 cases | 3+positive p16 cases | Total no. of cases (percentage %) |
|---|---|---|---|---|
| WD | 06 | 02 | 00 | 08 (34.7%) |
| MD | 12 | 01 | 02 | 15 (65.3%) |
| PD | 00 | 00 | 00 | 00 (0.00) |
| Total | 18 | 03 | 02 | 23 (100.0%) |

**Table 11:** p16 correlation with stage group. p16 expression was shown by stage I in which 2 out of 3 cases (66.6%) showed 1+ expression, Stage II showed 5 out of 9 cases (55.5%) with positive expression, of which 4 cases showed 1+ expression. In stage IVA, 5 out of 11 cases (45.5%) showed positive expression of which 3 cases showed 1+ expression. Stage IVB showed 2 out of 4 cases (50.0%) all of which showed 2+ expression and none of the 4 cases of stage III showed any p16 expression

| Stage group | Negative | P16 1+ | P16 2+ | Total |
|---|---|---|---|---|
| I | 01 | 02 | 00 | 03 |
| II | 04 | 04 | 01 | 09 |
| III | 04 | 00 | 00 | 04 |
| IVA | 06 | 03 | 02 | 11 |
| IVB | 02 | 00 | 02 | 04 |
| Total | 17 | 09 | 05 | 31 |

**Table 12:** Ki67 labelling index. Out of 78 cases, 34 (43.7%) showed intermediate expression (16 to 30%) followed by 24 (30.7%) cases with Ki67 expression of >=30% i. e. high grade, and 20 (25.6%) patients showed low grade of expression i. e. <=15%

| Grade | No. of cases | Percentage % |
|---|---|---|
| Low (<=15%) | 20 | 25.6 |
| Intermediate (16-30%) | 34 | 43.7 |
| High (>=30%) | 24 | 30.7 |
| Total | 78 | 100 |

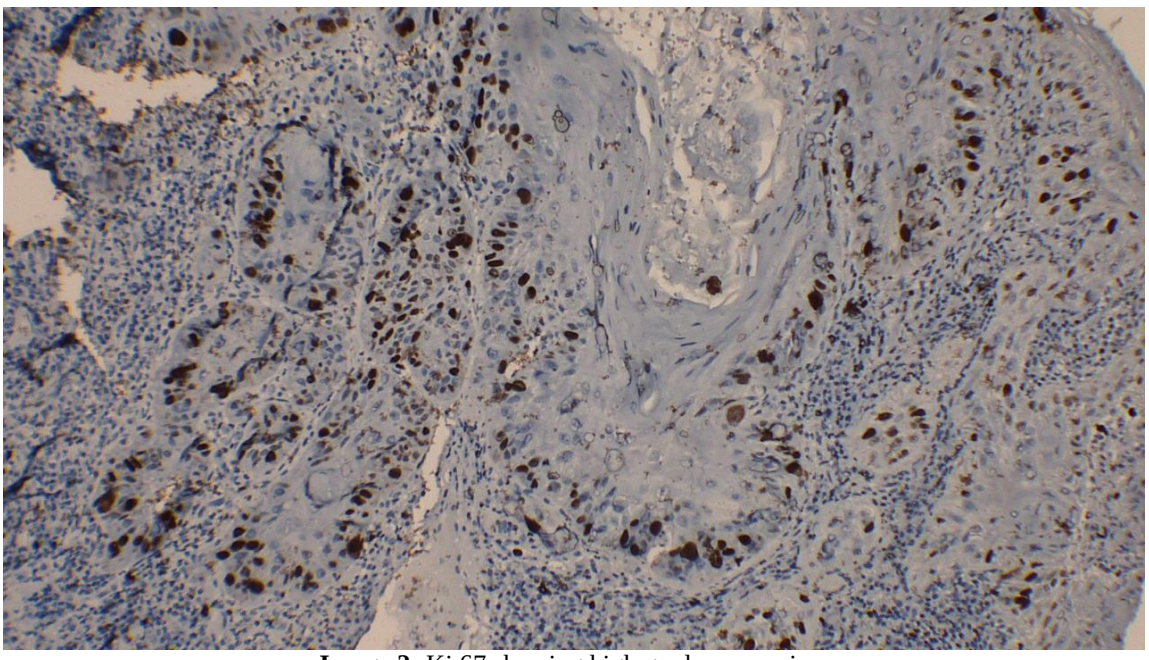

**Image 2:** Ki 67 showing high grade expression

**Table 13:** Ki67 expression correlated with the site. The majority of cases was from posterior GBS i. e.25 out of 78 cases (32.2%) of which 13 cases (52.0%) showed high Ki67 index, followed by cheek mucosa showed 23 cases (29.5%) of which 13 cases (56.5%) showed intermediate positivity, anterior GBS showed 21 cases (26.9%) of which 11 cases (52.3%) showed intermediate positivity.

| Site | Low | Intermediate | High | Total |
|---|---|---|---|---|
| Cheek mucosa | 05 | 13 | 05 | 23 (29.5%) |
| Retromolar area | 03 | 02 | 01 | 06 (7.6%) |
| Ant GBS | 05 | 11 | 05 | 21 (26.9%) |
| Post GBS | 04 | 08 | 13 | 25 (32.2%) |
| Lower lip | 03 | 00 | 00 | 03 (3.8%) |
| Total | 20 | 34 | 24 | 78 (100.0%) |

**Table 14:** Correlation of Ki67 expression with differentiation.16 cases out of 45 (35.5%) of moderately differentiated carcinomas and 16 out of 28 cases (57.1%) cases of well differentiated carcinomas showed intermediate Ki67 expression. Moderately differentiated carcinomas show more or less the same number of cases in all three. The only cases of dysplasia and poorly differentiated carcinoma showed high Ki67 expression.

| Histology | Low Ki67 | Intermediate Ki67 | High Ki67 | Total |
|---|---|---|---|---|
| Hyperplasia | 01 | 02 | 00 | 03 (3.8%) |
| Dysplasia | 00 | 0 | 01 | 01 (1.2%) |
| Well differentiated Carcinoma | 05 | 16 | 07 | 28 (35.8%) |
| Moderately differentiated Carcinoma | 14 | 16 | 15 | 45 (57.6%) |
| Poorly differentiated Carcinoma | 00 | 00 | 01 | 01 (1.2%) |
| Total | 20 | 34 | 24 | 78 (100.0%) |

**Table 15:** Ki67 index correlation with stage group. Stage IVA showed 11 out of 31 cases (35.4%) of which 9 (82%) cases showed intermediate Ki67 expression, followed by 9 out of 31 cases (29.0%) in Stage II of which 5 cases (55.5%) cases showed high Ki67 expression. Stage III and Stage IVB showed 4 cases (13.0%) of which all 4 cases of stage IVB showed high Ki67 expression while 3 out of 4 cases (75.0%) showed intermediate Ki67 expression. All 3 cases of stage I showed intermediate Ki 67 expression

| Stage Group | Cases showing low Ki67 | Cases showing intermediate Ki67 | Cases showing high Ki67 | TOTAL (%) |
|---|---|---|---|---|
| I | 00 | 03 | 00 | 03 (9.6%) |
| II | 03 | 01 | 05 | 09 (29.0%) |
| III | 00 | 03 | 01 | 04 (13.0%) |
| IVA | 00 | 09 | 02 | 11 (35.4%) |
| IVB | 00 | 00 | 04 | 04 (13.0%) |
| Total | 03 | 16 | 12 | 31 (100.0%) |

**Table 16:** The distribution of patients as per grading calculated by adding the group grade of both intensity and distribution of stained cells.48 (78.2%) out of 78 cases showed strong positive EGFR while 23 (29.6%) cases showed moderate expression. Only 7 (8.9%) cases showed weak expression.

| EGFR expression | No. of cases | Percentage % |
|---|---|---|
| 1-2 (weak) | 07 | 8.9 |
| 3-4 (moderate) | 23 | 29.6 |
| 5-6 (strong) | 48 | 61.5 |
| Total 78 | | 100.0 |

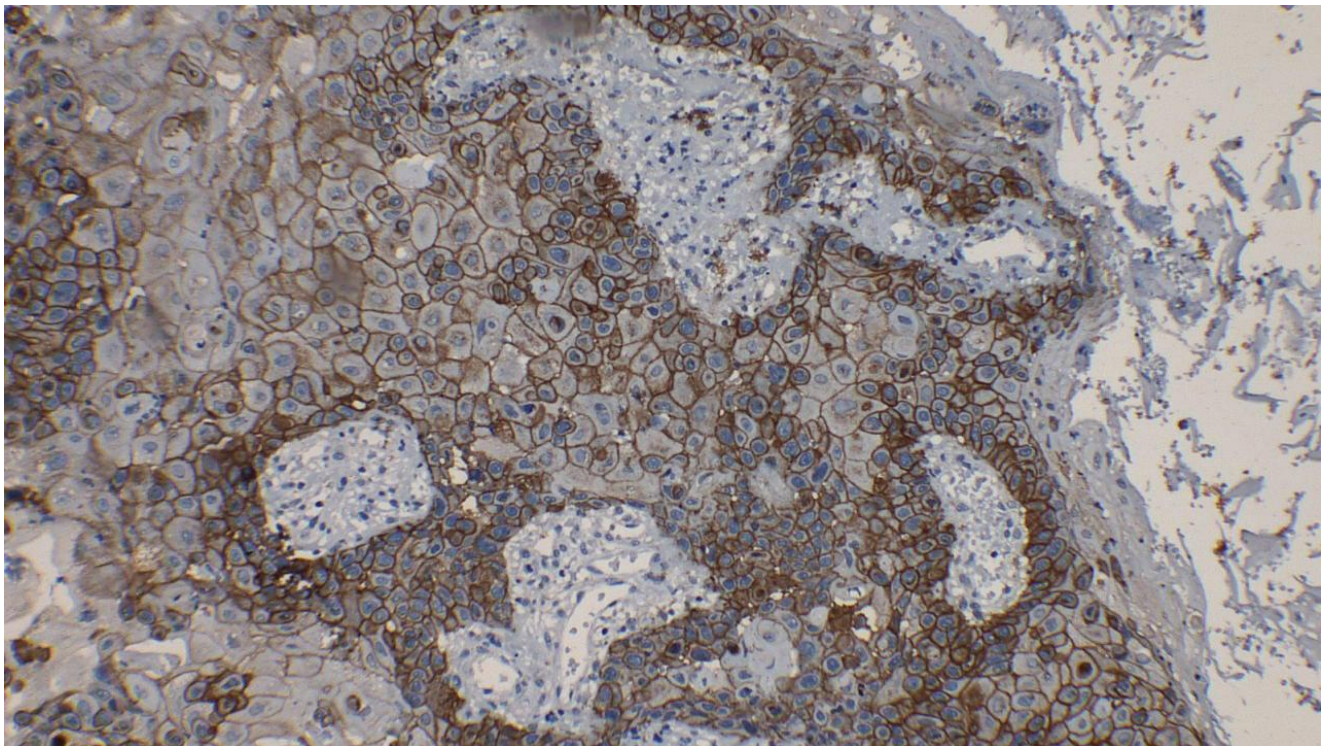

**Image 3:** Strong cytoplasmic and membranous EGFR expression.

**Table 17:** The association between site of lesion and expression of EGFR. Majority of the cases, 15 cases out of 25 cases (60.0%) in posterior GBS showed strong expression. While 17 cases out of 23 cases (73.9%) in cheek mucosa showed strong expression.13 cases out of 21 cases (61.9%) of anterior GBS showed strong expression.

| Site | Weak expression | Moderate expression | Strong expression | Total (%) |
|---|---|---|---|---|
| Cheek mucosa | 04 | 02 | 17 | 23 (29.5) |
| Retromolar area | 03 | 00 | 03 | 06 (7.6) |
| Ant GBS | 00 | 08 | 13 | 21 (26.9) |
| Post GBS | 00 | 10 | 15 | 25 (32.0) |
| Tongue | 00 | 03 | 00 | 03 (3.8) |
| Total | 07 | 23 | 48 | 78 (100.0) |

**Table 18:** EGFR grading in histologically differentiated cases is shown.37 of 45 cases (82.2%) of moderately differentiated carcinomas showed strong EGFR expression.25 of 28 cases of well differentiated carcinomas showed moderate and strong EGFR expression. Rest all cases showed almost similar EGFR expression. Moderate expression is seen maximum in well differentiated carcinomas cases i. e.14 out of 23 cases (60.8%) and strong expression was seen maximum in moderately differentiated carcinomas i. e.37 out of 48 cases (77.1%).

| Histology | Weak expression | Moderate expression | Strong expression | Total (%) |
|---|---|---|---|---|
| H | 02 | 01 | 00 | 03 (3.8) |
| D | 00 | 01 | 00 | 01 (1.4) |
| WD | 03 | 14 | 11 | 28 (35.8) |
| MD | 01 | 07 | 37 | 45 (57.6) |
| PD | 01 | 00 | 00 | 01 (1.4) |
| TOTAL | 07 | 23 | 48 | 78 (100.0) |

**Table 19:** Correlation of EGFR grade with stage group is shown.17 of 31 cases (54.8%) showed strong EGFR expression (5-6) followed by intermediate EGFR expression (3-4) in 11 (35.6%) cases and only 3 cases (9.6%) showed weak EGFR expression (1-2). Strong expression was seen in 8 out of 11 cases of IVA. Moderate expression is seen in 5 out of 9 cases (55.5%) in stage II.

| Stage group | Weak expression | Moderate expression | Strong expression | Total (%) |
|---|---|---|---|---|
| I | 00 | 02 | 01 | 03 (9.6) |
| II | 01 | 05 | 03 | 09 (29.0) |
| III | 00 | 01 | 03 | 04 (13.0) |
| IVA | 01 | 02 | 08 | 11 (35.4) |
| IVB | 01 | 01 | 02 | 04 (13.0) |
| Total | 03 (9.6%) | 11 (35.6%) | 17 (54.8%) | 31 (100.0) |

EGFR IHC:

- 91.1% showed moderate and strong EGFR expression
- 82.2% cases of moderately differentiated carcinoma showed strong EGFR expression.
- Moderate and strong expression of EGFR was seen most commonly in Stage Group IVA and Stage Group II

Ki67 IHC:

- 43.7% of all cases showed intermediate grade Ki67 LI's, followed by 30.7% with high grade.
- Maximum high and intermediate Ki67 expression was seen in MDSCC (39.7%) cases
- 35.4% cases of intermediate or high Ki67 LI was seen in Stage Group IVA followed by Stage Group II

## 4. Discussion

HPV enables over expression of p16 acting through inactivation of p53 and RB pathways. We found this in 31% cases. Pathak et al. stated for other cases, hypermethylation of the p16 promoter is likely cause of accumulation of p16 protein in cells. Pathak A et al found 54% cases with 2+ and 3+ but the staining intensity was low (1+ only) in most of our cases. [2, 6] Limited correlation in literature on relation with differentiation and stage group. We found maximum correlation with MD SCC and with Stage groups IVA & II. The mean Ki67 LI was 34.62 in this study vs 42.87% (Takkem et al) and 39.45% (Dwivedi et al). [3, 4]Intermediate and high expressions seen maximum in stage group IVA (35%) and MD SCC (42%) cases, suggesting a better correlation with differentiation rather than stage group. Grandis JR et al. have shown a high positive EGFR staining rate (92.3%) vs 91% in this study, with 63.4% of all cases exhibiting strong EGFR expression vs 61% in this study. [5]p16 positivity showed good correlation with moderate and strong EGFR expression and intermediate and high Ki67 LI's. May suggest that p16 positive OSCC act through EGFR/TRK pathway activation leading to increased rate of proliferation which is why EGFR and Ki67 is also important.

## 5. Conclusion

The three influences-etiological factor (p16/ HR HPV), EGFR (TRK activation) and Ki67 (proliferative rate). HPV infection plays a role in the pathogenesis of OSCC as shown by p16 surrogate expression in 31% cases. HPV causes activation of EGFR which in turn causes increased TRK activation resulting into RAS activation and in turn causes Increased transcription and cellular proliferation which is evident as increased expression of EGFR and Ki67 in this study.

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