Evaluation of p16 expression in oral and oropharyngeal squamous cell carcinoma

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INTRODUCTION

Oral and oropharyngeal cancers, are sixth most common cancers in the world, with >90% being squamous cell carcinomas (OSCC, OPSCC). HPV (Human Papilloma virus) is a major risk factor and can be assessed through p16 expression. HPv-positive cancers have a better prognosis, even with more advanced disease. p16 is also an independent favorable prognostic factor irrespective of HPV status.

The study evaluates p16 expression in these cancers and correlates it with age, gender, site/type of tumor and degree of differentiation. We have incorporated Lewis/modified Lewis criteria, to stratify them into prognostically favorable groups.

MATERIALS AND METHODS

This was an observational study, from July 2017 to June 2019 on a sample size of 70. It included all biopsies and
resected specimens received in the department of pathology from patients with clinically suspected oral (tongue, buccal mucosa, gingivobuccal sulcus, retromolar trigone, hard palate, and floor of mouth) and oropharyngeal (base of tongue, tonsil, vallecula, posterior pharyngeal wall, and soft palate) cancer. Relevant clinical details were taken. Ethical clearance has been obtained by the Institutional ethics committee.

All specimens/biopsies were fixed in 10% neutral buffered formalin (NBF) and subjected to routine processing for paraffin embedding. Two sections were cut from each block, one (4-5 µ) stained with Hematoxylin and Eosin (H&E) while the other (3 µ) on poly-L-lysine slide for p16 immunohistochemical analysis. The study included all cases of squamous cell carcinomas (SCC), dysplasia, and carcinoma in situ. The SCC’s were categorized as per the W.H.O classification and the conventional SCCs were graded from well to poorly differentiated. Exclusion criteria were inadequate biopsy and benign lesions.

Immunohistochemical staining for p16INK4a was performed with both positive and negative controls, using Biogenex Lifesystems Histology Kit (G175-405 Clone), in accordance with the manufacturer’s instructions.

**Interpretation of p16 staining:** Sections showing both complete (nuclear and cytoplasmic) and/or partial (only cytoplasmic) staining in >= 5% cells were considered positive. Sections showing no staining or staining in <5% cells were considered as negative. The p16-positive slides were graded semi-quantitatively in a quartile manner into four grades based on percentage of cells showing positive immunostaining as Grade1 (up to 25%), Grade 2 (26-50%), Grade 3 (51-75%), and Grade 4 (>75%). They were further assessed for degree of confluence (defined as group of 10 contiguous cells with positive immunostaining) in a semi-quantitative quartile manner (up to 25% grade 1, 26-50% grade 2, 51-75% grade 3 and >75% grade 4). We further applied the Lewis criteria (>75% p16-positive cells OR > 50% p16-positive cells with >25% confluence) and modified Lewis criteria (>75% p16-positive cells OR 26-75% positive cells with >75% confluence) to identify patients, who might have an increased overall survival and better prognosis.

The data was entered in Microsoft Excel master sheet and analyzed using Statistical Package for Social Sciences (SPSS) version 17.0 software. A value of $P < 0.05$ was taken as significant.

**RESULTS**

A total 75 cases were evaluated, of which 70 cases were included in the study. Of the five cases excluded, two were benign lesions and three were inadequate biopsies. The 70 cases included histologically diagnosed cases of OSCC (44/70, 63%) and OPSCC (26/70, 37%).

The age of the patients in the study ranged from 31 to 95 years with a mean of 56.9 ± 15.6 years, and a peak between 40 to 60 years (47.1%). Among OSCC, the age ranged from 31 to 95 years with a mean of 53.8 ± 16.1 years and a peak between 40 to 60 years age (52.3%). Whereas in OPSCC, the age ranged from 35 to 85 years with a mean of 62.0 ± 13.5 years and a peak above 60 years (57.7%).

Most of the patients were males 47/70 (67%), with females accounting for 23/70 (33%) cases and showing a male:female ratio of 2:1. Patients with OSCC comprised of 27 males (61.4%) and 17 females (38.6%) with a male:female ratio of 1.6:1 and those with OPSCC comprised of 20 males (76.9%) and 6 females (23.1%) with a male:female ratio of 3.3:1.

The most common sub-site in OSCC was tongue 16/44 (36.4%), followed by buccal mucosa 13/44 (29.5%) and GBS 9/44 (20.5%). The other sites were hard palate 3/44 (6.8%), floor of mouth 2/44 (4.5%) and retromolar trigone 1/44 (2.3%). Among the OPSCCs, the most common sub-site involved was base of the tongue 10/26 (38.5%), followed by tonsil 9/26 (34.6%) and vallecula 4/26 (15.4%). The other sites were soft palate 2/26 (7.7%) and posterior pharyngeal wall 1/26 (3.8%).

Histological grading was done for all cases. Of the 70 cases, 41 (58.6%) were MDSCC, 18 (35.7%) WDSCC, and 5 (7.1%) PDSCC. There were two (2.9%) cases each of Adenosquamous carcinoma, Basaloid SCC, and Dysplasia.

In OSCCs, 25/44 (56.8%) were MDSCC, 13/44 (29.5%) WDSCC, 4/44 (9.1%) PDSCC, and 2/44 (4.6%) were Adenosquamous carcinoma. In OPSCCs, 16/26 (61.5%) were MDSCC, 5/26 (19.2%) WDSCC, 1/26 (3.8%) was PDSCC and two cases each (7.7%) were Basaloid SCC and dysplasia.

The p16 IHC showed that 24/70 (34%) cases were negative, while 46/70 (66%) were positive. Of the 44 cases of OSCC, 32 (72.7%) were p16 positive, while 12 (27.3%) were negative. Of the 26 cases of OPSCC, 14 (53.8%) were p16 positive, while 12 (46.2%) were negative.
Overall (both OSCC and OPSCC) p16 positivity was seen in a younger subset of patients (mean age 54.4 years), than p16 negative ones (mean age 61.9 years). Though, p16-positive cases had a lower mean age (52 yrs and 60.9 yrs) as compared to p16-negative cases (58.7 yrs and 63.3 yrs), it was not statistically significant. Overall, p16 positivity was seen in 39.1% (9/23) female and 78.7% (37/47) male patients. Thus, a statistical significance (P = 0.001) was seen between male gender and p16 positivity.

In OSCC, p16 positivity was seen in 35.3% (6/17) female and 96.3% (26/27) male patients. Thus, a statistical significance (P < 0.001) could be seen between the male gender and p16 positivity. Whereas in OPSCC, p16 positivity was seen in 50% (3/6) female patients and 55% (11/20) male patients. However, this was not statistically significant.

When histological differentiation and p16 was considered among OSCC, p16 positivity was seen in 22/25 (88%) MDSCC, 2/4 (50%) PDSCC, 7/13 (53.9%) WDSCC, and 1/2 (50%) of adenosquamous carcinoma. Among OPSCC, p16 positivity was seen in 8/16 (50%) MDSCC, 1/1 (100%) PDSCC, 4/5 (80%) WDSCC, and 1/2 (50%) Basaloid SCC. There was no statistical correlation between degree of differentiation and p16.

When sub-site evaluation of p16 positivity was considered, among OSCC it was seen that 13/16 (81.3%) from the tongue were p16 positive, followed by 8/13 (61.5%) in buccal mucosa and 5/9 (55.6%) in GBS. All cases from hard palate 3/3 (100%), retromolar trigone 1/1 (100%), and floor of mouth 2/2 (100%) were p16 positive. In OPSCC, 7/10 (70%) cases from base of tongue were p16 positive, followed by 4/9 (44.4%) in tonsil, 1/2 (50%) in soft palate, and 1/4 (25%) in vallecula. The single case from PPW 1/1 (100%) was p16 positive.

The quartile method of IHC grading was applied to the p16-positive cases and when confluence grading was applied to the same it was seen that 9/32 (28%) OSCC and 5/14 (35.7%) OPSCC fit in Lewis criteria [Figures 2 and 3]. Also, 11/32 (34%) OSCC and 7/14 (50%) OPSCC fit in modified Lewis criteria. [Table 1].

**DISCUSSION**

Oral and Oropharyngeal cancers together are the sixth most common cancers in the world, accounting for 4% of all malignancies in males and 2% in females. Oral cancer is among the top three cancers in India, while OPSCC represents 10–15% of all head and neck cancers. The risk factors associated are alcoholism, smoking, tobacco, and HPV. In India, more than 90% of oral cancer cases...
p16 positivity was seen in 32/44 (72.7%) OSCCs. Pathak et al.\textsuperscript{[8]} and Dragomir et al.\textsuperscript{[21]} showed p16 positivity in majority of cases (31/50, 62% and 22/34, 64.7% respectively). Present study noted p16 positivity in younger patients (52 years) like Salas et al.\textsuperscript{[23]} (48.67 years); as compared to p16 negative ones (current study 58.7 years, Salas et al. 63.71 years). A statistically significant association between male gender and p16 positivity has also been noted. Sritippho et al.\textsuperscript{[28]} also showed a higher p16 positivity in males (6/19, 31.6%) when compared to females (4/22, 18.2%). However, Babiker et al.\textsuperscript{[3]} noted that p16 positivity was higher in females (13/24, 54.2%) than males (27/76, 35.5%). Both the studies were statistically insignificant.

When histological differentiation was considered, there was no association between p16 and degree of differentiation in the current study. Babiker et al.\textsuperscript{[3]} noted statistically significant p16 expression in 12/22 (54.5%) cases of WDSCC, with lower association in MDSCC (16/34, 47.1%) and PDSCC (12/44, 27.3%). However, Sritippho et al.\textsuperscript{[28]} noted p16 positivity in 6/16 (37.5%) cases of WDSCC, while MDSCC and PDSCC cases did not show p16 positivity. Also, 1/2 (50%) cases of Adenosquamous carcinoma showed p16 positivity in the present study, similar to findings of Masand et al.\textsuperscript{[24]} (2/4, 50% p16 positive). When subsite of OSCC and p16 expression are considered, the current study showed no statistical significance. However, the study done by Zafereo et al.\textsuperscript{[20]} showed a statistical correlation with p16 expression being predominant in cases from the tongue [Table 2]. The other studies (Sritippho et al., Yang et al.)\textsuperscript{[4,28]} showed no statistical significance. However, on review of results in all the studies, it is noted that a major proportion of cases from tongue, hard palate and buccal mucosa show p16 expression. [Table 2].

### Oropharyngeal SCC: OPSCCs, were mostly seen in the base of tongue 10/26 (38.5%) and tonsil 9/26 (34.6%),
p16 positivity was seen in 14/26 (53.8%) cases of OPSCC, which is comparable to findings by Shinohara et al.29 (29/53, 54.7%) and lower than those of Samuel et al.30 (48/81, 59.3%). p16-positive patients were younger (60.9 yrs) when compared to p16-negative patients (63.3 yrs). This is similar to findings of Shinohara et al.29 and Barasch et al.31 where the mean age was lower in p16-positive patients (61 yrs and 53.5 yrs, respectively) in contrast to p16-negative ones (67.5 yrs and 62 yrs, respectively). However, unlike the present study, both these studies showed statistical significance.30,26 Other findings of Fischer CA et al.31 and Broglie (p16 positive 60.4 yrs and 63 yrs, p16 negative 58 yrs and 60 yrs, respectively) et al.31 were not in concordance with the present study, since they showed a higher mean age in p16-positive patients. Gender wise, p16 positivity was seen in a higher number of males 3/6 (50%), when compared to females 11/20 (55%). This is similar to findings of Barasch et al.31 with higher p16 positivity in males (35/57, 61.4%) compared to females (13/24, 54.2%). Whereas, Shinohara S et al.,30 noted that p16 positivity was higher in female (8/11, 72.7%) than male (21/42, 50%) patients. Both the studies had shown no statistical significance.29,26

When histological differentiation was considered, the present study had a higher number of WDSCC (4/5, 80%) and MDSCC (8/16, 50%) showing p16 positivity. These observations were unlike those seen by Weinberger et al.28 where, p16 positivity was seen in zero cases of WDSCC, 5/53 (9.4%) MDSCC, and 14/45 (31.1%) PDSCC. They also found this to be statistically significant, unlike the present study. While, 1/2 (50%) cases of Basaloid SCC showed p16 positivity, Chernock et al.29 showed 8/12 (66.7%) cases of Basaloid SCC to be p16 positive. When subset and p16 expression was taken into account, all three studies (Barasch et al.,31 Broglie et al.,31 current study) could not find any statistical significance [Table 3]. However, what has been similar in all studies is that a good proportion of cases from the base of tongue and tonsil showed p16 expression. [Table 3].

It was seen that 14/46 (30.4%) cases could be included in Lewis criteria (OSCC—9/32, 28%; OPSCC—5/14, 35.7%), while 18/46 (39.1%) cases could be included in modified Lewis criteria (OSCC—11/32, 34%, OPSCC—7/14, 50%). Though the present study hasn’t evaluated the follow up of patients, it is known that patients have an overall improved survival and better response to treatment.31 Applying these criteria is simple, but additional research with larger numbers, evaluating follow up, response to therapy and survival is needed to determine the prognosis of these subsets of patients.

To conclude, OSCC and OPSCC are common in the age of 40–60 yrs with a male predominance. p16 positivity was seen in 72.7% cases of OSCC and 53.8% cases of OPSCC. It is seen in a younger age group, more in males and has no association with degree of differentiation. p16 expression was more often noted in tongue, buccal mucosa, hard palate in OSCCs, and among OPSCCs in base of tongue and tonsil. p16 is a simple and inexpensive prognostic marker which can be routinely evaluated in these cancers.
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Conflicts of interest
There are no conflicts of interest.

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