RESEARCH ARTICLE

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Relevance of Partial p16 Immunostaining in Oral Squamous Cell Carcinoma and Co- Relation with HPV DNA Status

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Abstract

Introduction: Immunostaining criteria for p16 positivity in oropharyngeal squamous cell carcinoma have been laid down by College of American Pathologists (CAP) and the American Society of Clinical Oncology (ASCO). The staining should be of moderate to strong intensity seen in 70 percent of the tumor cells. Recent studies have pointed out that a small minority of cases are missed using p16 as the surrogate marker at above mentioned cut off. By convention the same criteria have been used for oral squamous cell carcinoma. **Material and Methods:** The authors revisited the results of their previous study where immunohistochemistry for p16 was found to be positive by AJCC criteria in 139 out of 800 cases of oral squamous cell carcinoma. For this study, all the p16 immunonegative cases (by AJCC criteria) were analysed again for partial staining patterns, defined for this study as cases with 50-75% cells showing 2+/3+ intensity of nuclear p16 immunostaining and for basal predominant pattern of immunostaining. These cases were subjected to HPV DNA PCR. **Results:** Out of the 661/800 cases found to be negative for p16 immunohistochemistry, a total of 34/800(4.25%) showed partial staining based on the criterion of 50-75% cells showing p16 immunostaining intensity of 2/3+.The basal predominant pattern of immunostaining for p16 was seen in 43/800 (5.38%) cases. When these cases were subjected to HPV DNA analysis, 11/34 (32.35%) of the cases showing partial staining and 02/43 (4.7%) of the cases showing basal predominant pattern of p16 immunostaining were found to be HPV-DNA positive. **Conclusion:** The inclusion of partial immunostaining patterns of p16 in HPV analysis of oral squamous cell carcinoma can improve our understanding of HPV driven oral squamous cell carcinoma.

Keywords: Oral squamous cell carcinoma- Human papilloma virus- partial p16 immunostaining patterns

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Introduction

Immunostaining criteria for p16 positivity in oropharyngeal squamous cell carcinoma have been laid down by College of American Pathologists (CAP) and the American Society of Clinical Oncology (ASCO). The staining should be of moderate to strong intensity seen in 70 percent of the tumor cells. Recent studies have pointed out that a small minority of cases are missed using p16 as the surrogate marker at above mentioned cut off. Partial staining on p16 immunohistochemistry (more than 50% and less than 70%), a basal cell predominant positivity in the tumor nests and cases with discordant morphology (ie non keratinizing type but p16 immunonegative) have been investigated in many studies (Lewis et al., 2012; Shinn et al., 2021) In countries where HPV is more prevalent in the oropharyngeal squamous cell carcinomas like United States (70% OPSCC are HPV related tumors) a reclassification scheme has been proposed (Shinn et al., 2021). According to this scheme HPV mRNA RTPCR testing when applied to equivocal p16 immunostained cases (50 to 70% of tumor cells moderate to strongly positive) and to morphologically discordant cases (non-keratinizing but p16 immunonegative) was able to identify 14 more cases out of a cohort of 467 as HPV related tumors. These tumors would have been missed on using p16 as surrogate marker for HPV (Shinn et al., 2021).

The cut off for p16 immunostaining has been a matter of much debate but numerous studies have now proved that for oropharyngeal squamous cell carcinoma a higher cut off of 70 percent is able to identify a relatively pure cohort of HPV DNA PCR positive cases. However, 5 to 10% of these cases would still be HPV DNA PCR negative (Shinn et al., 2021; Machiels et al., 2020)). The percentage of HPV related tumors amongst the

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oropharyngeal carcinoma cases varies widely depending on the geographic location, however the global estimate is around 30 to 35% (Machiels et al., 2020).

In a previous study, the authors have studied the demography of p16 positive and HPV DNA PCR positive squamous cell carcinoma cases of oral cavity (Naz et al., 2022). In this study 139/800 (17.37%) cases were p16 immunopositive and out of these 104/139 (74.8%) cases were also positive for HPV DNA PCR (Naz et al., 2022).

In the current study the authors aim to study the same cohort for the relevance of partial staining pattern (50% to 75% staining) and basal cell predominant positivity for p16 immunohistochemistry.

Materials and Methods

The authors revisited the results of their previous study where immunohistochemistry for p16 was found to be positive by AJCC criteria in 139 out of 800 cases of oral squamous cell carcinoma (Naz et al., 2022).

In that study, 800 cases of OSCC (Oral squamous cell carcinoma) were included. Informed consent was taken and a detailed questionnaire was administered regarding demographic details and habits. Immunohistochemistry for p16 protein using CINtec histology kit (E6H4 clone,Roche diagnostics) was done and the AJCC criteria for p16 immunopositivity were followed. Moderate to strong nuclear staining in more than 75% of the cells was taken as the criteria for positivity. Formalin Fixed Paraffin Embedded tissue (FFPE) was utilized for DNA extraction using A2352 (Promega, Madison, WI, USA) Promega Reliaprep (FFPE gDNA Miniprep system). The DNA yield was evaluated by Nanodrop (Thermofischer). Hybribio- 14 High-risk HPV with 16/18 Genotyping Real time PCR kit was utilized for HPV detection. The kit could broadly classify the HPV detected into three categories-HPV 16,HPV 18, and Other HPV types. However the kit could not specify which of the other HPV is present in the sample.

For this study, all the p16 immunonegative cases (by AJCC criteria) were analysed again for partial staining patterns, defined for this study as cases with 50-75% cells showing 2+/3+ intensity of nuclear p16 immunostaining and for basal predominant pattern of immunostaining. These cases were subjected to HPV DNA PCR.

The study was cleared by the Institute Ethics Committee for Human Research Ref. no. IEC-286/01.06.2018, RP-17/2018. The research grant was provided by Ministry of Health and Family Welfare (Department of Health Research): Grant number- File No.R.12013/03/2018-HR.

Results

Out of the 661/800 cases found to be negative for p16 immunohistochemistry, a total of 34/800(4.25%) showed partial staining based on the criterion of 50-75% cells showing p16 immunostaining intensity of 2/3+ (Figure 1, Table 1). Interestingly all the cases showing partial staining had an intensity of 2+ (moderate) and none had 3+ intensity.

The basal predominant pattern of immunostaining for p16 was seen in 43/800 (5.38%) cases (Figure 1).

When these cases were subjected to HPV DNA analysis, 11/34 (32.35%) of the cases showing partial staining and 02/43 (4.7%) of the cases showing basal predominant pattern of p16 immunostaining were found to be HPV-DNA positive. Out of these 11 cases, 6 cases were positive for HPV-16, 4 cases were positive for HPV -18 and one case positive for both. None of the cases showed positivity for Other HPV types. Out of the two cases with basal predominant pattern of immunopositivity one case each was positive for HPV 16 and HPV 18 alone.

The demographic details of the cases showing partial and basal predominant pattern of immunostaining has been

Table 1. Summary of the p16 Immunohistochemistry, HPV DNA Findings and Demographic Features of Partial Positive and Basal Predominant Cases

S.No		
1	Total number of cases	800
2	Cases showing p16 positivity by AJCC criteria	139/800(17.38%)
3	Cases showing partial p16 positivity	34/800 (4.25%)
4	Partial p16 positive cases showing HPV DNA positivity	11/34 (32.35%)
5	Cases showing basal predominant pattern of p16 positivity.	43/800 (5.37%)
6	Basal predominant p16 positive cases positive for HPV DNA Demographic features	2/43 (4.7%)
7	Partial positive cases	Basal predominant cases
	Male:31/34 (91.2%) Female:03/34(8.8%) Smoker:30/34 (88.2%) Tobacco chewing: 22/34 (64.7%)	Male:39/43 (90.7%) Female:4/43 (9.3%) Smoker: 39/43 (90.7%) Tobacco chewing: 29/43(67.4%)
	Site :	Site:
	Anterior two third of tongue:14/34 (41,2%) Buccal mucosa:12/34 (35.3%) Soft Palate:03/34 (8.8%) Hard palate:01/34 (2.9%) Floor of mouth:04/34 (11.7%)	Anterior two third of tongue:18/43 (41.9%) Buccal mucosa:16/43 (37.2%) Soft Palate:03/43 (6.9%) Hard palate:04/43 (9.3%) Floor of mouth:02/43 (4.6%)

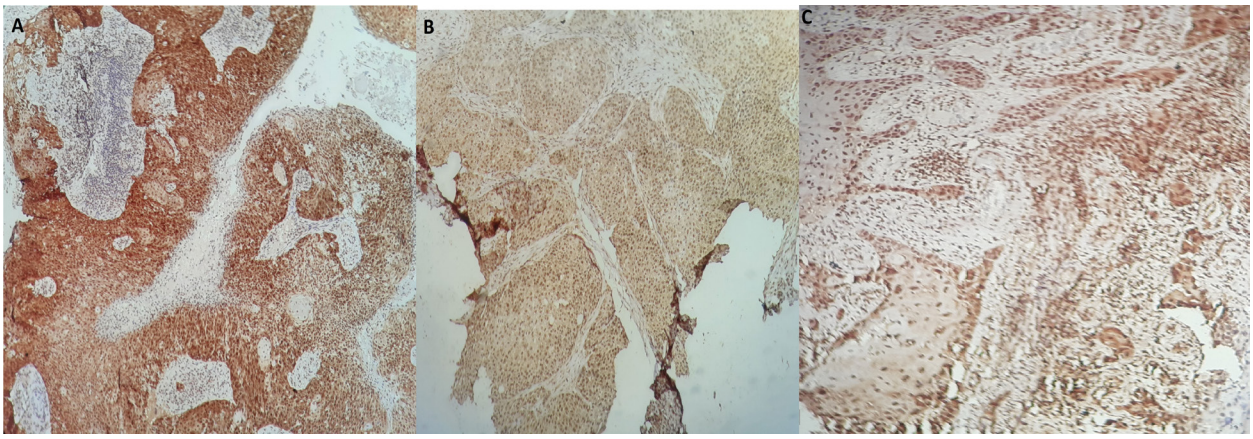

Figure 1. Immunohistochemistry for p16 Pprotein –A (100x) –Positive by AJCC criteria (>75% strong nuclear positivity), B (100x)- Partial positivity (50 to 75% moderate nuclear positivity), C (400x)-basal predominant pattern of p16 immunopositivity.

summarised in (Table 1). Hence if these two patterns were included in the screening protocol of oral squamous cell carcinoma for HPV a total of 13 more cases would have been detected with HPV infection.

Discussion

Based on histomorphology, the oropharyngeal squamous cell carcinomas have been classified as Keratinizing, non-keratinizing and non-keratinizing with maturation (Chernock et al., 2012). A study conducted in the year 2009 showed that non- keratinizing oropharyngeal squamous cell carcinoma were 47% out of 118 cases (Chernock et al., 2009). These cases were more common in the younger age group and were more likely to be HPV and p16 positive (Gondim et al., 2016). However outside the oropharynx, non-keratinizing morphology is rare.

Hence the results of studies on oropharynx cannot be extrapolated to oral cavity tumors where HPV DNA positivity is less. The histopathological features suggestive of p16 immunopositivity in oral cavity tumors are totally different from those in oropharyngeal tumors (Gayatree et al.,2020). The reason for this could be that non HPV pathway for p16 immunopositivity is more important in the oral cavity. The cut off for p16 immunopositivity in the oropharyngeal tumors has been well characterized and the same cut off has been used for oral cavity tumors too (Naz et al., 2022; Gayatree et al., 2020). The prognostic significance of p16 immunopositivity in oral cavity squamous cell carcinoma has long been a controversial topic. In oral squamous cell carcinoma, p16 immunopositivity has been associated with a worse clinical outcome in some studies (Maléřová et al., 2020; Saghravanian et al. 2016). On the other hand, a tissue microarray based study has reported longer disease free survival in such cases (Satgunaseelan et al., 2016). One possible explanation for the variability in the results could be the difference in interpretation of partial staining cases. Hence it is imperative to find out the implications of partial p16 immunostaining on HPV DNA analysis so that the actual number of HPV related tumors are evaluated for their impact on the clinical outcome (Ryu et al., 2017).

Our study highlights the importance of assessing the partial staining patterns in oral cavity squamous cell carcinoma. If we include the partial staining of p16 and basal predominant p16 immunopositivity in tumor assessment, the percentage of HPV related oral cavity squamous cell carcinoma would increase to 19.0% (139+13=152 out of 800).

The cases showing HPV DNA positivity in the absence of strictly defined cut off of 75% p16 immunopositivity should be included in the category of HPV driven carcinomas. The entire paradigm of viral integration with the host DNA in the head and neck squamous cell carcinomas has undergone a change in the last few years. There are three scenarios with respect to the viral and host genome in the oral cavity squamous cell carcinomas- episomal viral genome, viral genome integrated to the host genome and finally a mixture of both integrated and episomal genomes. Recent work has suggested that the last category has been wrongly characterised as integrated genome. It actually consists of virus–human hybrid episomes. Hence by conventional wisdom the cases with 75% p16 immunopositivity are the cases with genomically integrated HPV, however there are other scenarios where the virus human hybrid episomes can impart imperfect p16 immunostaining (Morgan et al., 2017).

We believe that the inclusion of partial immunostaining patterns of p16 in HPV analysis of oral squamous cell carcinoma can improve our understanding of HPV driven oral squamous cell carcinoma. The limitations of this assumption are obvious and have been tried but both of them have their own set of problems.

Future studies with Fluorescence In Situ Hybridization and mRNA analysis on the small but significant subset of p16 partially staining cases and basal predominant p16 positive cases can shed new light on the role of HPV in oral cavity squamous cell carcinoma.

Author Contribution Statement

Farhat Naz: Conceptualization, designed, investigation, data curation, analysis and writing of original manuscript draft. Hitesh Verma: methodology and manuscript

review. Nadeem Tanveer: Conceptualization, designed, data analysis, and manuscript draft. Sudheer Arava: methodology and reviewing manuscript. Aanchal Kakkar: methodology and reviewing manuscript. Amar Ranjan: manuscript review, Anita Chopra: manuscript review, Pranay Tanwar: methodology, analysis and reviewing manuscript.

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Ethical Clearance

The study was given clearance by the Institute Ethics Committee for Human Research Ref. no. IEC-286/01.06.2018, RP-17/2018

Declaration of Competing Interest

All the authors declare that they have no competing and conflict of interest.

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