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P16 Immunohistochemistry as a Surrogate Marker for High-Risk HPV in Oral Squamous Cell Carcinoma

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ABSTRACT

BACKGROUND: HPV-related oral squamous cell carcinoma (OSCC), a unique form of head and neck cancer, primarily affects the oropharynx. The primary causative agent is high-risk HPV types, predominantly HPV 16. HPV's E6 and E7 genes are central to HPV-induced cancer development. The E7 oncogene causes strong p16 expression in HPV-positive tumor cells. p16 immunohistochemistry is considered a preferred test for predicting outcomes in OSCC since it is cheaper, easier than other HPV tests, widely available, easily interpreted, and accurately predicts patient outcomes.
OBJECTIVES: This study aims to evaluate HPV expression in oral squamous cell carcinoma (OSCC) and to assess its correlation with different histological grades of OSCC using p16 immunohistochemistry. This study also aims to correlate between the patterns of p16 expression with the various histological grades of OSCC.
METHODOLOGY: This retrospective study analysed 30 paraffin-embedded OSCC cases over one year, including well, moderately, and poorly differentiated subtypes. Sections were stained with p16 monoclonal antibody (Clone JC8). Three observers evaluated p16 expression, assessing both positivity rates and staining patterns across different histological grades.
RESULTS: Among 30 OSCC cases, overall p16 positivity was 70% which was seen increasing with tumor grade: well-differentiated 66.7%, moderately-differentiated 69.2%, poorly-differentiated 100%, showing progressively stronger and more diffuse staining.
CONCLUSION: Our study showed an association between HPV and OSCC using p16 immunohistochemistry, also revealing distinct predominant staining patterns in various histological grades of OSCC. Hence, our study suggests that p16 immunohistochemistry can be considered a potential surrogate marker for HPV infection in OSCC, especially in resource limited settings.

Keywords: High Risk HPV, Oral Squamous Cell Carcinoma, p16 Immunohistochemistry, Surrogate Marker, Association between HPV and Oral squamous cell carcinoma, Original Research Article in Pathology

INTRODUCTION

Oral and pharyngeal cancers rank as the sixth most common cancers worldwide, with a particularly high burden in South and Southeast Asia (1). Over 90% of oral cancers are squamous cell carcinoma (SCC) (2), which can arise at any site in the oral cavity, most commonly the tongue and floor of the mouth. Established risk factors include tobacco use (smoked and smokeless forms, especially betel quid) and alcohol consumption, emphasizing the role of lifestyle in oral squamous cell carcinoma (OSCC) pathogenesis (3). Recently, Human papillomavirus (HPV) has emerged as an important etiological factor in a subset of OSCCs. HPV prevalence varies across head and neck sites, with reports of approximately 59% in oral cavity tumors, 43% in pharyngeal tumors, and 33% in laryngeal tumors (3). HPV are small, circular double-stranded DNA viruses that integrate into host cells and disrupt normal cellular processes (4). The viral oncogenes E6 and E7 drive HPV-induced carcinogenesis by interfering with tumor suppressor proteins, including p53 and pRb (5).

The protein p16, encoded by the CDKN2A gene, functions as a tumor suppressor involved in cell cycle regulation. Normally, p16 inhibits cyclin D-dependent kinases (CDK4 and CDK6), maintaining Rb in a hypophosphorylated state, preventing its dissociation from E2F transcription factor, and blocking progression into S phase (6). In normal cells, p16 expression is minimal and nearly undetectable by immunohistochemistry (IHC). However, E7-mediated transformation leads to overexpression of p16 in HPV-positive tumor cells, allowing detection via IHC (4). p16 positivity may also arise from HPV-independent mechanisms, such as Rb gene alterations or other Rb pathway mutations (6). Upregulated p16 expression is strongly associated with HPV infection and serves as a valuable diagnostic and prognostic marker (7). p16 is also referred to as multiple tumor suppressor 1 (MTS1), cyclin-dependent kinase inhibitor 2A (CDKN2A), or p16 INK4a (6).

While the prognostic and diagnostic significance of HPV in OSCC remains debated, p16 immunohistochemistry is considered a reliable surrogate marker of high-risk HPV infection. This study is particularly relevant in the Indian population, where OSCC contributes substantially to cancer-related morbidity and mortality. The role of high-risk HPV and p16 expression in OSCC remains underexplored regionally, necessitating generation of local data. Therefore, this study was undertaken to evaluate p16 as a potential diagnostic marker in OSCC and its correlation with different histological grades.

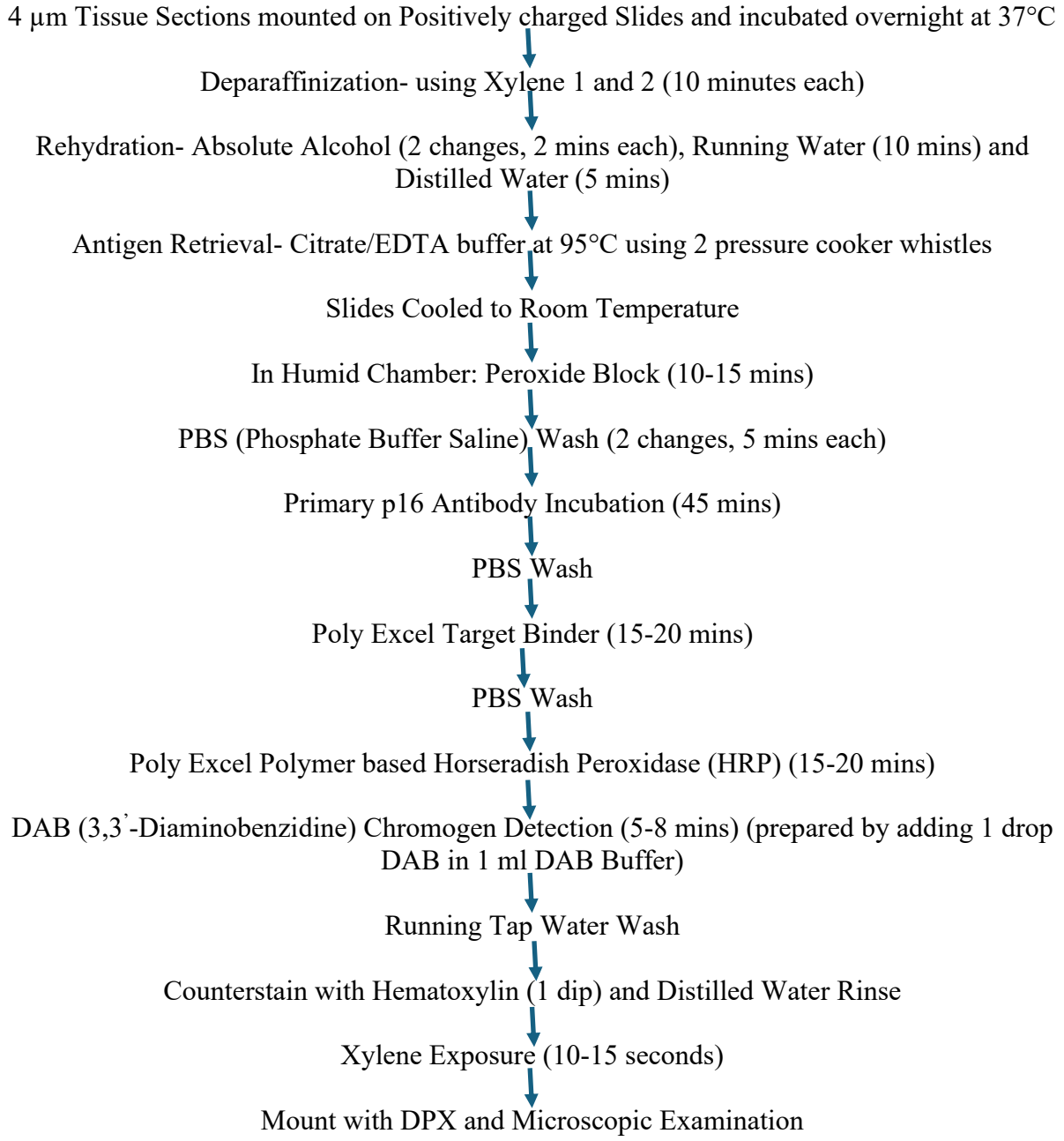
MATERIALS & METHODS

This retrospective study was conducted in the Department of Pathology in a Government Medical College, Karnataka, after obtaining ethical clearance from the Institutional Ethics Committee. A total of 30 formalin-fixed, paraffin-embedded (FFPE) tissue blocks of OSCC, retrieved from the archives of the Histopathology section over a 1-year period (January 2024-December 2024) were included. Resected cases of primary OSCC where blocks were available were included while recurrent tumors, previously treated tumors and cases where blocks were not available or sufficient tissue was not available for cutting were excluded. The cohort comprised of 15 well-differentiated, 13 moderately differentiated, and 2 poorly differentiated OSCC cases.

Sections of 4 µm thickness were cut from each block and mounted on Aminopropyltriethoxysilane (APES) coated positive slides. Immunohistochemical staining was performed using Pathn Situ p16 INK4A (clone JC8) mouse monoclonal antibody at a 1:50 dilution, following standard protocols. Positive controls (HPV-positive cervical carcinoma) and negative controls (without primary antibody) were included.

Steps in p16 Immunohistochemistry Staining:

P16 INK4A is affinity purified and diluted in antibody diluent with 1% bovine serum albumin (BSA) and 0.05% of sodium azide (NaN_3). The dilution used was 1:50. (8)



P16 INK4A stains both the cytoplasm and nucleus (block staining). College of American Pathologists (CAP) guidelines define p16 IHC as positive when there is ≥70% of tumor cells exhibiting both nuclear and cytoplasmic staining of moderate to strong intensity. (9)

Two aspects of p16 expression, measured by immunohistochemistry, were assessed:

1) The proportion of cases showing p16 positivity, and
2) The staining pattern of p16 across different histological grades of OSCC.

Table I: Criteria for Evaluating p16 Staining were based on reference (10):

Grades	Grades
0	No Staining
1	Singly Dispersed Cells
2	Patchy Staining
3	Diffuse Staining

All slides were independently evaluated by three pathologists, with discrepancies resolved by consensus to minimize inter-observer variability. Given the small sample size, descriptive statistics using frequency and percentage distribution were used.

RESULT

A total of 30 histologically confirmed cases of Oral Squamous Cell Carcinoma (OSCC) were analysed using p16 immunohistochemistry (IHC). The cohort included 15 well-differentiated (WD), 13 moderately-differentiated (MD), and 2 poorly-differentiated (PD) OSCC cases.

The median age of presentation was 55 years, with most patients belonging to middle-aged (43.3%) and older adult groups (40.0%). A marked male predominance (83.3%) was noted, with a male-to-female ratio of 5:1. The buccal mucosa was the most frequently involved site (60%), followed by the tongue (16.7%), gingivobuccal sulcus (13.3%), and lip (10%). The predominant clinical presentation was ulceroinfiltrative growth (50.0%), followed by ulceroproliferative (36.7%) and proliferative growth patterns (13.3%).

Table II: Site Distribution of OSCC

Site	Number of cases	Percentage (%)
Buccal Mucosa	18	60.0
Tongue	5	16.7
Gingivobuccal sulcus	4	13.3
Lip	3	10.0

p16 IHC Analysis:

Out of the 30 OSCC cases, 70% (21 out of 30) showed nuclear and cytoplasmic p16 positivity.

- **Well-differentiated OSCC:** 10 out of 15 cases (66.7%) demonstrated p16 positivity. The staining was typically with mild to moderate staining intensity and was focal to patchy. Most of these cases showed a Grade 1 staining pattern.
- **Moderately-differentiated OSCC:** 9 out of 13 cases (69.2%) were positive for p16. The staining in these cases was more extensive than in well-differentiated OSCC, with moderate intensity and involving larger clusters of tumor cells. Majority of them showed a Grade 2 staining pattern, characterized by widespread nuclear and cytoplasmic positivity.
- **Poorly-differentiated OSCC:** Both cases (2/2, 100%) showed strong and diffuse p16 positivity. The staining intensity in these tumors were moderate and uniform, and staining pattern was diffuse throughout the tumor nests. These cases were categorized as Grade 3 p16 staining pattern, indicating high expression levels across nearly all tumor cells.

Table III: Association between Histological Grade and p16 Positivity in OSCC

Histological Grade	Total Cases (n)	p16 Positive n (%)	p16 Negative n(%)
Well Differentiated	15	10 (66.7%)	5 (33.3%)
Moderately Differentiated	13	9 (69.2%)	4 (30.8%)
Poorly Differentiated	2	2 (100%)	0 (0%)
Total	30	21 (70.0%)	9 (30.0%)

A Fisher–Freeman–Halton exact test was performed to assess the association between tumor grade and p16 positivity. Although p16 positivity increased from 66.7% in

well-differentiated OSCC to 100% in poorly differentiated OSCC, the difference was not statistically significant (P =1.00). The Pearson Chi-square test (χ^2=0.94, df=2, P =0.625) gave a similar result. These findings suggest that while there is a descriptive trend toward increased p16 expression in higher-grade OSCC, the present sample size is insufficient to establish a statistically significant correlation.

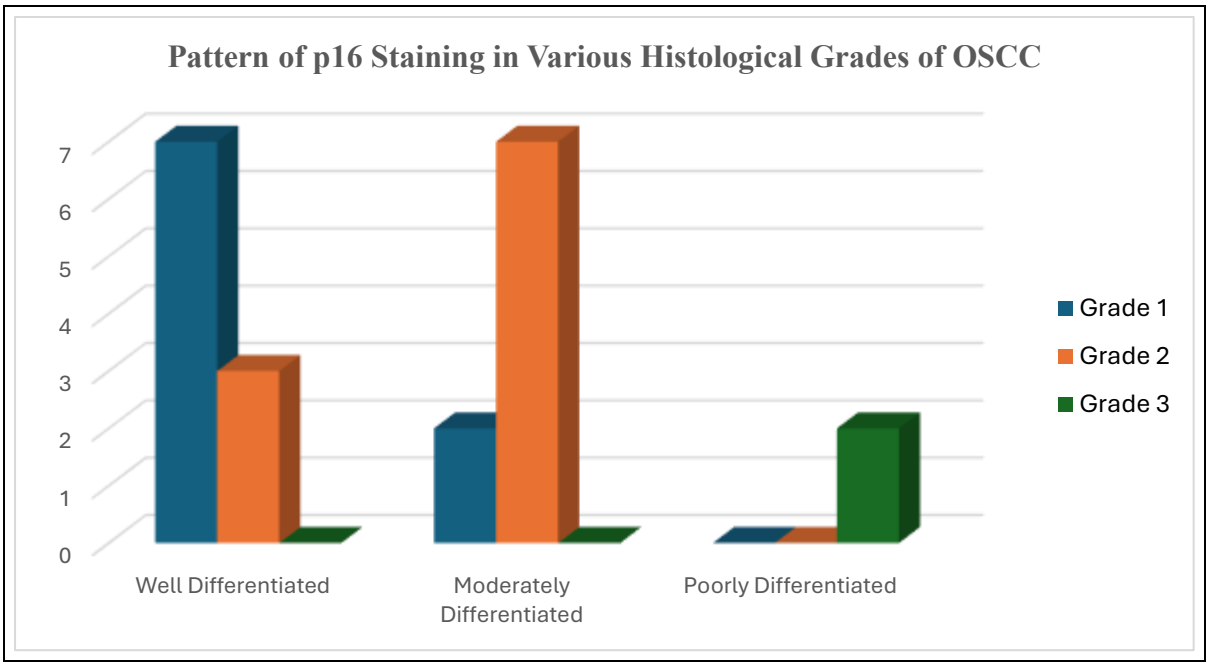


Fig I: Pattern of p16 staining in various histological grades of OSCC.

The pattern of p16 immunohistochemical expression in various histological grades of OSCC showed that in well-differentiated OSCC, p16 positivity was predominantly observed in Grade 1 staining with fewer cases showing Grade 2 staining, whereas moderately-differentiated OSCC demonstrated a higher proportion of Grade 2 staining with fewer cases showing Grade 1 staining. No Grade 3 staining was observed in both well-differentiated and moderately-differentiated OSCC cases. Poorly-differentiated OSCC showed predominantly Grade 3 p16 staining, while Grade 1 and Grade 2 expression were minimal. Overall, Figure I illustrates an increasing trend of p16 staining pattern and expression with increasing histological grade of OSCC.

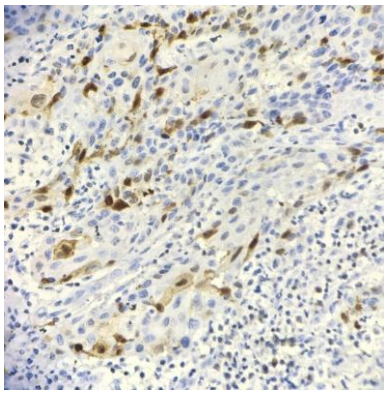


Fig II: Grade 1 p16 staining

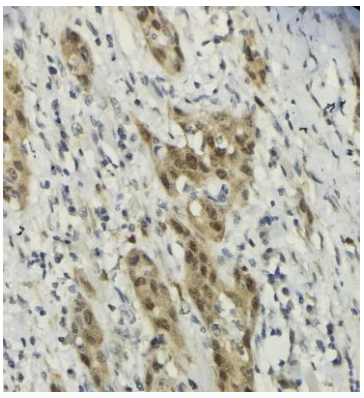


Fig III: Grade 2 p16 staining

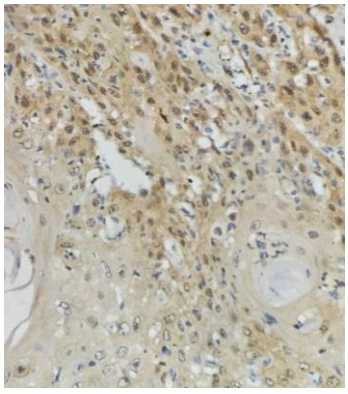


Fig IV: Grade 3 p16 staining

Hence, the intensity of p16 immunostaining was mild to moderate in well-differentiated OSCC (Grade 1), and moderate in both moderately and poorly differentiated tumors

(Grades 2 and 3). While staining intensity did not markedly differ between Grades 2 and 3, the distribution of positive cells increased with grade, with Grade 3 tumors showing more diffuse and widespread positivity across tumor nests. This indicates a biological trend toward increasing p16 expression with tumor de-differentiation.

In summary, p16 positivity was detected in 70% of OSCC cases, with a clear trend toward stronger and more diffuse expression in higher-grade tumors.

DISCUSSION

This study aimed to assess the role of p16 immunohistochemistry (IHC) as a surrogate marker for high-risk HPV infection in OSCC and to correlate its expression with histological tumor grade. A notable finding was that 70% of the OSCC cases showed p16 positivity and a biological trend toward increased expression with tumor de-differentiation was evident.

The observed pattern supports the theory that p16 overexpression is associated with HPV-mediated oncogenesis. p16 is a cyclin-dependent kinase inhibitor that becomes overexpressed in HPV-driven carcinomas, particularly as a consequence of E7 oncoprotein-mediated inactivation of the retinoblastoma (Rb) protein. This inactivation triggers compensatory upregulation of p16, making it a reliable surrogate marker for oncogenic HPV activity, particularly HPV-16. This biological basis renders p16 a practical and cost-effective surrogate marker, especially in resource-limited settings.

Our findings are consistent with and align with several previous studies. Patil et al. [4] (2014) reported a similar trend, with p16 positivity noted in 86.66% of OSCC of which p16 positivity was seen in 70% of well-, 90% of moderately-, and 100% of poorly-differentiated OSCC cases. They also observed an increasing frequency of diffuse staining in higher-grade tumors, suggesting a correlation between tumor aggressiveness and HPV involvement. Verma et al. [11] (2019) expanded on this understanding by showing that p16 expression could be observed even in patients without traditional risk factors like tobacco or alcohol use, indicating HPV as an independent etiological factor. This reinforces the relevance of p16 IHC, particularly in younger patients or those lacking conventional exposures. A 2025 study by Goswami et al. [12] also supports our findings, showing increased p16 expression across the histological spectrum-from 50% in well-differentiated to 90% in poorly differentiated OSCC. Additionally, they noted a progressive rise in p16 expression from dysplasia to carcinoma, implying that p16 may also serve as a marker for early malignant transformation.

However, the interpretation of p16 overexpression must be cautious. El-Naggar et al. [13] (WHO 2017) reported that p16 may be upregulated even in HPV-negative tumors due to non-viral mechanisms like Rb mutations. Thus, while p16 is a reliable screening tool, confirmatory testing with PCR or in-situ hybridization (ISH) is recommended in ambiguous cases.

Sritippho et al. [14,15] (2015, 2016) similarly highlighted that while p16 overexpression is frequent in HPV-related OSCC, its diagnostic accuracy improves when used in conjunction with molecular testing. They emphasized its significance in younger populations and its association with high-risk HPV types, particularly HPV-16.

Geographical variation in HPV prevalence is another crucial consideration. Studies show that South Indian populations have a higher prevalence of HPV-positive OSCC compared to Western and Japanese cohorts. Patil et al. noted 67% HPV positivity in South India, compared to 15% in Western India, 23% in Japan, and 8–20% in the USA. These disparities likely reflect differences in sexual practices, diagnostic methodologies, and anatomical subsite involvement.

Interestingly, in our study, the buccal mucosa was the most common site of OSCC- contrasting with Western populations where the oropharynx is often

the primary site for HPV-associated carcinomas. This is likely due to the regional prevalence of risk factors like betel quid and tobacco chewing. Rathish et al. [16] (2020) and Pimolbutr et al. [17] (2025) corroborate that HPV-associated OSCC in Asia frequently involves the buccal mucosa, warranting more localized studies on p16 expression in these contexts.

CONCLUSION

In summary, our findings validate the role of p16 IHC in assessing HPV-associated OSCC. While not definitive for HPV detection, p16 is a valuable surrogate marker for high-risk HPV involvement, particularly when supported by clinical and histopathological features. Its practical advantages- ease of use, affordability, and interpretability- make it suitable as a first-line diagnostic tool in under-resourced healthcare systems.

Limitation And Future Scope

One limitation of this study includes a small sample size, particularly in poorly differentiated group, and also the absence of confirmatory molecular testing (PCR or ISH) for HPV diagnosis. In future, larger multicentric studies with diverse populations are required to validate the observed descriptive trend, which can also be combined with p16 and HPV DNA/RNA assays. Correlation of p16 expression with clinical outcomes (recurrence, survival) should be explored to establish prognostic value.

Declaration by Authors

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Conflict of Interest: The authors declare that they have no conflicts of interest in this study.

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