

RESEARCH ARTICLE

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Predictive Value of Anti- E6 Oncoprotein (High Risk- Human Papilloma Virus) and p16 Ink4a for Detecting HPV in Oral Epithelial Dysplasia

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Abstract

Aim: The aim of this study was to evaluate the expression of human papillomavirus (HPV) in oral potentially malignant disorders (OPMD) and to examine the association of HPV in histological grades of dysplasia using p16 and Anti-E6 oncoprotein immunohistochemistry (IHC). **Subjects and methods:** This study focused on clinically diagnosed oral potentially malignant disorders. Clinical parameters such as age, gender, habits, occupation, duration, site, and the type of the lesions were examined and the incisional biopsy was done on the selected cases for the histopathological diagnosis. Selected cases of OPMDs were screened immunohistochemically for HPV 16 and HPV 18 (high-risk group) positivity using p16INK4a and Anti-E6 oncoprotein. The immunohistochemical p16 expression was evaluated based on (a) percentage of p16 positive cases and (b) pattern of p16 staining in various grades of OPMD. **Results:** Anti-E6 oncoprotein (HR-HPV) expression level was only detected in 11 cases (37%), and positive expression of p16 was found in three cases (10%), with variation in cell proportion and intensity. Subsequently, the association between p16 expression level and clinicopathological characteristic factors was analyzed and a significant association was found between age and histopathology. **Conclusion:** There was an association between HPV and OPMD. Both biomarker tests, HPV E6 and p16 immunocytochemistry had a specific role in the detection of HR-HPV. Anti-E6 immunocytochemistry can be a valuable test with higher specificity for HPV DNA detection in oral epithelial dysplasia without losing sensitivity.

Keywords: Oral epithelial dysplasia- OPMD, HR HPV- p16INK4a- anti- E6 oncoprotein

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Introduction

Oral cancer is the sixth most common malignancy worldwide with a survival rate that depends on staging and grading the tumour (Petersen, 2009). The most common malignancy is oral squamous cell carcinoma and it usually occurs as a multi-step process. According to the GLOBOCAN report 2020, India ranks at 2nd top position in terms of oral cancers prevalence in the world, with an annual incidence of 14.7% (Mathur et al., 2020).

The current model of oral carcinogenesis postulates transformation from a normal to potentially malignant and invasive carcinoma phenotype. Histologically, the transition begins from benign hyperplasia to various degrees of dysplasia (low and high grade) and finally into oral squamous cell carcinoma (Akhter et al., 2011; Ranganathan et al., 2019). Multiple factors are involved in the occurrence and progression of a precursor lesion to malignancy. Early identification and detection of the factors in assessing the high-risk patients greatly helps in the prevention of malignant transformation (Messadi,

2013; Poh et al., 2011). The factors determining malignant transformation usually depend on lateral border of tongue, floor of mouth, type of the lesion (verrucous, non-homogenous, and PVL), HPV infection, and Staging and Grading (high grade). Establishing a risk prediction model using clinical, histopathological, and immunohistochemical parameters and applying it on OPMDs may be useful in the prevention of malignancy (Silverman, 2001; Swango, 1996).

There is still substantial controversy over the precise contribution of human papillomaviruses (HPV) to the emergence of premalignant lesions and oral squamous cell carcinoma (OSCC). Smoking in its various forms, heavy alcohol consumption, and the chewing of areca nuts and betel quid are well-known primary environmental risk factors for OSCC. Contrary to a recent and contentious study by Johns Hopkins, cancer is a negative accident caused, in around two thirds of instances, by random mutations occurring during DNA replication in normal and non-cancerous stem cells, which is unrelated to genetics or ecological factors. In contrast, high-risk human

papillomaviruses (HR-HPV) have recently been revealed to have a stronger connection with OSCC in the Western population, accounting for about 15-20% of individuals with the disease (Chocolatewala et al., 2009).

Human papillomaviruses are small, circular double-stranded deoxyribonucleic acid (DNA) viruses that belong to the papillomaviridae family (Hafed et al., 2012). Over 130 HPV known types, HPV-16 and 18 are the most commonly detected high-risk types. The epitheliotropism of HPVs is a distinctive feature. Expression of the viral oncoproteins E6 and E7 results in cell cycle dysregulation by inactivating p53 and pRb, respectively, and is a key factor in HPV-associated carcinogenesis; which results in cell growth and malignant transformation. Telomerase erosion and dysregulated cellular proliferation are symptoms of ubiquitination caused by E6 and the degradation of p53. Because HPV E6 has the ability to interact with the telomerase complex and trigger the production of telomerase reverse transcriptase, which activates telomerase and leads to cellular immortalization, the degradation is significantly reduced. Other cellular proteins important for cell-cycle progression are the targets of E7. In order to initiate the stationary (S) phase of the cell cycle, E7 is known to bind with p21 and target it for ubiquitin-proteosomal destruction. E6 and E7 are known to trigger the Wnt signaling pathway, which prevents catenin from being phosphorylated and degraded by proteases. This prevents expression of cyclin D1, which starts the gap-1(G1) phase of the cell cycle. Since high-risk HPV genotypes' E6 and E7 oncoproteins have the ability to mediate the malignant transformation of infected keratinocytes by deactivating cellular p53 and pRb tumor suppressor pathways, HPV may either play an oncogenic or a co-oncogenic role in some HPV-related precancerous and cancerous epithelial neoplasms (Machado et al., 2010).

Hence, this study focused on evaluating the expression of HPV in OPMD and correlating the clinicopathological association of HPV in histological grades of dysplasia using p16 and Anti-E6 oncoprotein

Materials and Methods

A total of 30 formalin-fixed paraffin-embedded biopsied samples, histopathologically diagnosed as OPMD, were retrieved from the Department of Oral and Maxillofacial Pathology, CSI College Of Dental Sciences and Research, Madurai, India. Serial sections of 3-4µm were taken, one section was subjected to Haematoxylin and Eosin staining to ascertain the histological grades (WHO recent classification, 2017; Ranganathan et al., 2019) while the consecutive sections were subjected to p16 and Anti-E6 oncoprotein IHC analysis.

Out of the 30 cases of oral epithelial dysplasia, nine cases were Mild Dysplasia (Chocolatewala et al., 2019), 17 cases were Moderate Dysplasia [Group II], and four cases were Severe Dysplasia [Group III].

The procedure for p16 immunohistochemistry (IHC) is elucidated in Flowchart I. Commercial mouse monoclonal antibody for p16INK4a protein and Anti-E6 oncoprotein (NextGen Bio) was used. Positive control slides of

cervical carcinoma (Figure 1) harboring HPV were taken to ascertain the validity of the IHC kit and the accuracy of the technique. For negative control, the primary antibodies were omitted. The presence of brown precipitate at the site of cytoplasm, nucleus, or both were indicative of p16 and Anti-E6 oncoprotein positive immunoreactivity regardless of staining intensity (Bradley et al., 2006).

In order to reduce inter-observer bias, the slides were viewed under a light microscope by three pathologists in a blinded manner. The results were determined using student t-test for unconsolidated data and the chi-square test for raw data. The Bland-Altman Plot and ROC- Receiver Operating Characteristics were used to evaluate the diagnostic accuracy (sensitivity and specificity) of the tests.

Results

In total, 30 cases of OPMD were evaluated. The results revealed positive p16 in three cases (10%) out of 30 with variation in cell proportion and intensity (Figure 2). On the other, Anti-E6 oncoprotein (HR-HPV) was positive for 11 cases (36%). The positive cases and their association between various clinical parameters are listed in Table 1. However, no significant relation was observed in various clinical parameters and immunostaining of p16 anti-E6 oncoprotein.

Histopathologically the expression levels of E6 and p16 were higher in severe and moderate dysplasia; the differences were statistically significant ($P < 0.033$); and the Chi-Square value was 4.565

Prediction of high-risk HPV presence Using IHC p16 and Anti E6

The results of the analyses of using histologic features either alone or combined with Anti-E6 and p16 expressions to predict HPV presence in oral epithelial dysplasia are shown in Table 2, 3, and 4.

Histologic features along with the Anti- E6 oncoprotein in all the groups (Group 1, 2 and 3) combined (i.e. mitotoid and apoptotic cell(s) diffusely or focally present were 90.91% sensitive) (95% CI: 58.72%, 99.77%) and 94.74% specific (95% CI: 73.97%, 98.87 %). Anti-E6 oncoprotein showed a positive predictive value of 36.67.% and a

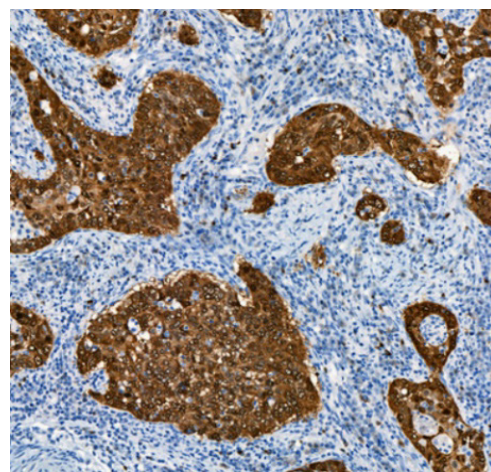


Figure 1. Positive Control (Cervical Carcinoma)

Table 1. Immunohistochemical Analysis of p16 and Anti E6 among Different Clinicopathological Parameters with the p-value

Characteristic	ANTI E6 ONCOPROTIEN POSITIVE	ANTI E6 ONCOPROTIEN NEGATIVE	P-Value	IHC p16 POSITIVE	IHC p16 Negative	P-Value
All patients (n=30)						
Gender						
Female	1	0		1	0	
Male	10	19	0.367 NS	2	27	0.100 NS
Age (yr)						
>=60	1	10		1	11	
<60	10	9	0.023 SIG	2	16	1.0 NS
Lesion site						
Other sites	9	18		2	25	
Lateral/ventral tongue	2	1	0.537 NS	1	2	0.280 NS
Lesion type						
Homogenous	10	18		2	21	
Non-homogenous	1	1	0.723 NS	1	0	0.125 NS
Colour						
White	9	18		2	25	
Red / Speckled	2	1	0.537 NS	1	2	0.280 NS
Smoking						
Never	1	1		1	1	
Past and present	10	17		2	23	
Unknown	0	1	0.693 NS	0	3	0.135 NS
Alcohol intake						
Never	1	2		1	2	
Past and present	10	15		2	22	
Unknown	0	2	0.524 NS	0	3	0.329 NS
Histopathological diagnosis						
Mild	0	9		1	13	
Moderate	9	8		1	11	
Severe	2	2	0.024 SIG	1	3	0.559 NS

negative predictive value of 90.91% in predicting the presence of high-risk HPV with an accuracy of 93.33%.

Whereas histologic features in p16 in all the groups were 18.8% sensitive (95% CI: 4.33%, 77.2%) and 80.00% specific (95% CI: 78.88 %, 99.89 %), with a positive predictive value of 33.33% and negative predictive value of 64.00% in predicting the presence of high-risk HPV with an accuracy of 58.06%.

Constructing The Limits of Agreement for IHC p16 And Anti E6 Oncoprotein

The rho (the power of relationship) value between p16 and Histopathology is 0.185 and 0.856 for Anti E6 oncoprotein; both have a good agreement (Figure 3 and 4).

The Bland Altman plot depicts a perfect line of

agreement that gives a diagnostic accuracy of -0.98 and +0.98 for the Histopathology and IHC p16 respectively and an accuracy of 0.515 and +0.515 for the Histopathology and IHC Anti E6 oncoprotein respectively.

Testing the diagnostic accuracy using ROC curve

The area under the curve for p16 is 0.7559, representing a 70% chance that the model is able to distinguish between histopathology and IHC p16. Thus, it is moderately accurate for a p16 test to detect HR-HPV (Figure 5). Whereas for Anti E6 oncoprotein, the area under the curve is 0.9707, which represents 90% chances that the model is able to distinguish between the histopathology and IHC Anti E6 oncoprotein. Thus, for an Anti-E6 test to detect HR-HPV, it is highly accurate (Figure 6). Anti-E6

Table 2. IHC p16 Prediction Vs Histopathological Analysis

Positivity	True Positive	False Positive
Positive	2	1
Negative	4	23
	False Negative	True Negative

Table 3. IHC Anti-E6 Oncoprotein Prediction Vs Histopathological Analysis

Positivity	True Positive	False Positive
Positive	10	1
Negative	1	18
	False Negative	True Negative

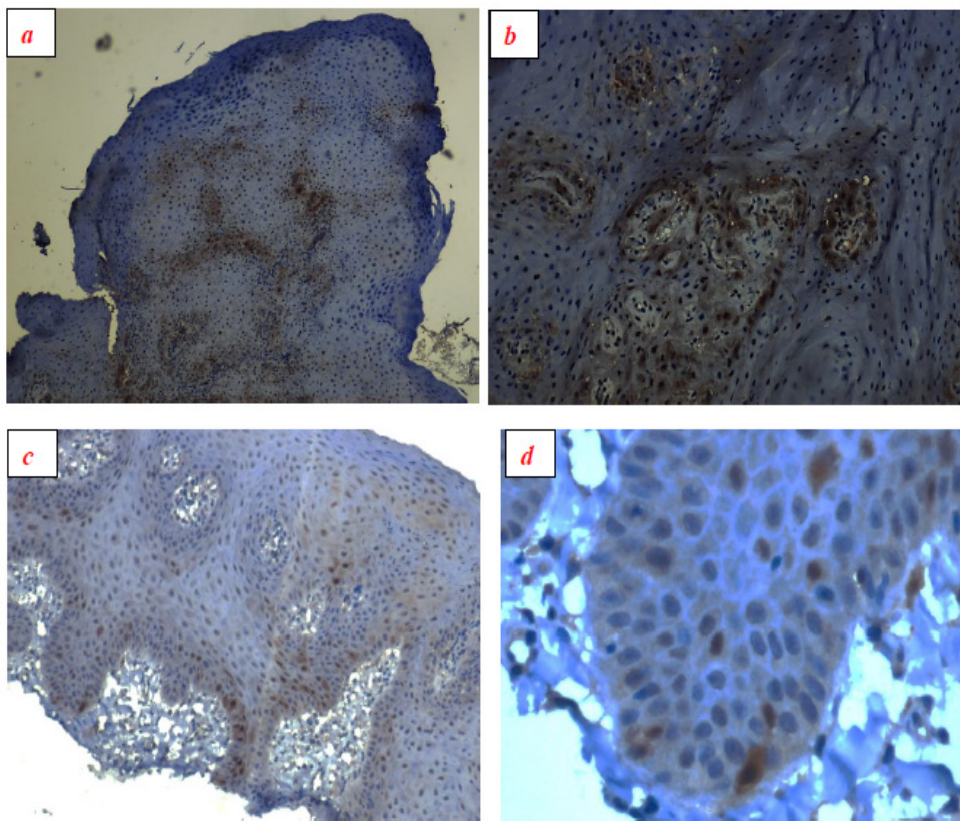


Figure 2. Immunostaining of Anti E6 (a and b) Oncoprotein and p16 (c and d) in Oral Dysplasia

has a higher diagnostic accuracy than that of p16INK4a in detecting the HR-HPV.

Discussion

In this analysis, we discovered that higher cases of oral epithelial dysplasia were associated with high-risk groups, with 11 of the 30 cases exhibiting immunopositivity for

E6 oncoprotein HPV 16/18.

Our results are not consistent with Liu et al., (2021) who investigated verrucous hyperplasia and verrucous cancer and reported a 0.3 percent immunopositivity in verrucous hyperplasia lesions. They suggested that this might be caused by either low HPV 16/18 E6 oncoprotein sensitivity, insufficient HPV 16/18 E6 protein expression, or involvement of low risk E6 oncoprotein types negative

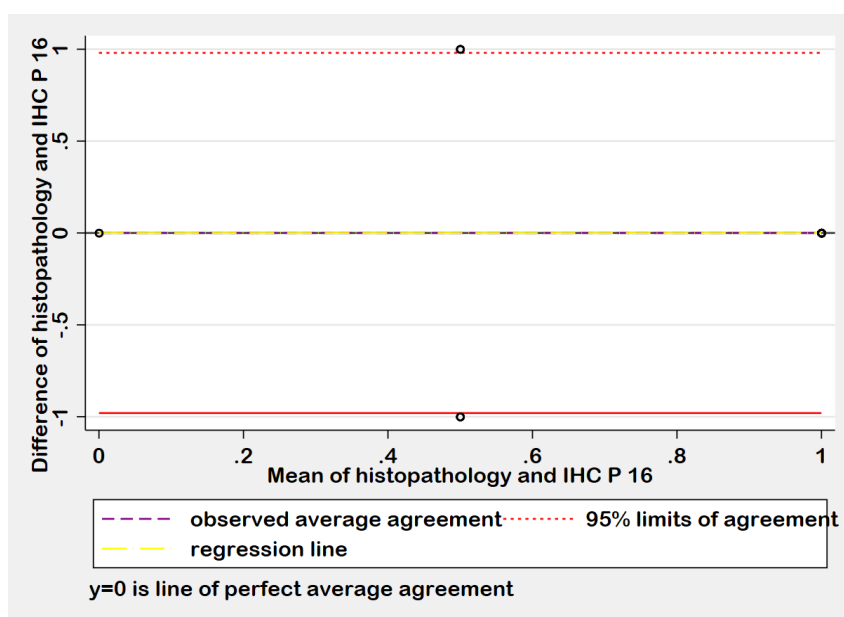


Figure 3. Bland-Altman Plot for IHC p16. The given curve depicts perfect line of agreement gives a diagnostic accuracy of -0.98 and +0.98 for the Histopathology and IHC p16

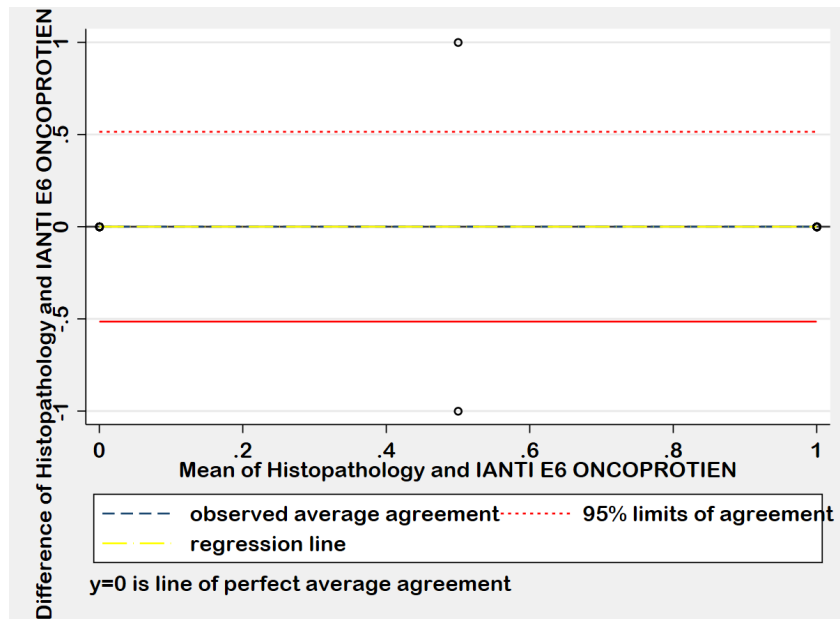


Figure 4. Bland-Altman Plot for IHC Anti E6. The given curve depicts perfect line of agreement gives a diagnostic accuracy of -0.515 and +0.515 for the Histopathology and IHC Anti E6 oncoprotein.

in generating verrucous hyperplasia.

The findings are likewise consistent with those of Cohen et al., (2018) who used a polymerase chain reaction to identify HPV 16/18 in samples of oral verrucous carcinoma and oral verrucous hyperplasia (PCR). The presence of HPV DNA in oral cancer tissue and that of high-risk HPV viruses and altered healthy oral epithelial cells support the idea that HPV has a role as an etiological agent in oral cancer. In light of this, the current investigation was done to check for the presence of HPV types 16 and 18 in oral epithelial dysplasia.

A significant change in OPMD and OSCC incidence was because of a decrease in the number of cases

associated with tobacco, while new cases were due to HPV. The etiopathogenesis of squamous cell carcinoma is important as HPV-associated OSCC and OPMD have higher curing rates than those associated with tobacco and alcohol risk factors (Cohen et al., 2018). Unfortunately, approximately 2/3 of lesions were identified at an advanced stage, which affected treatment options, requiring more complex therapy, and increasing the morbidity of treatment and cost of care. It is expected that management of OPMD and early-stage squamous cell carcinoma leads to a better prognosis (Rao et al., 2018). Although most OSCC cases are expected to be preceded by OPMD, it is not known whether OPMD arises from

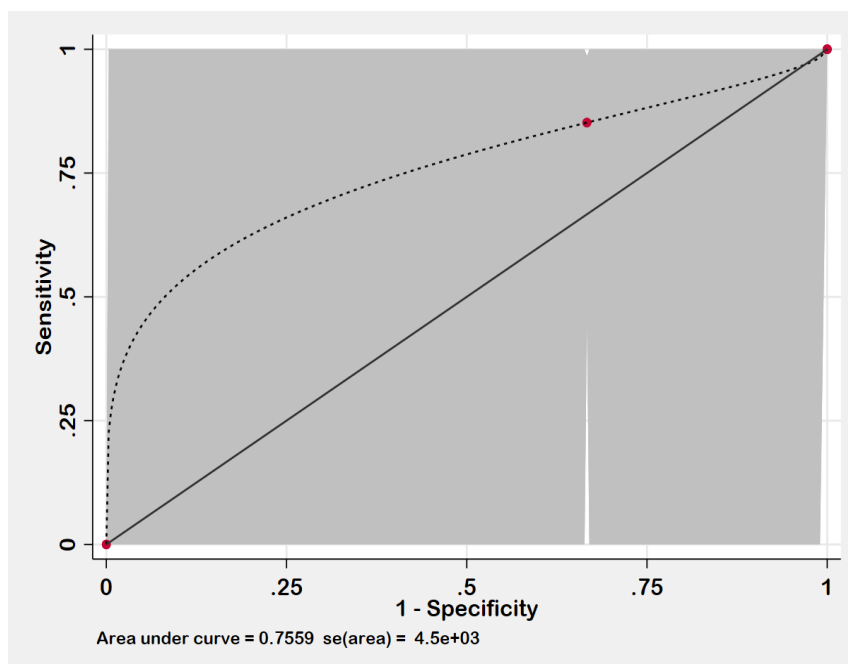


Figure 5. Receiver Operating Curve for IHC p16. The area under the curve for p16 is 0.7559, representing a 70% chance that the model is able to distinguish between the histopathology and IHC p16

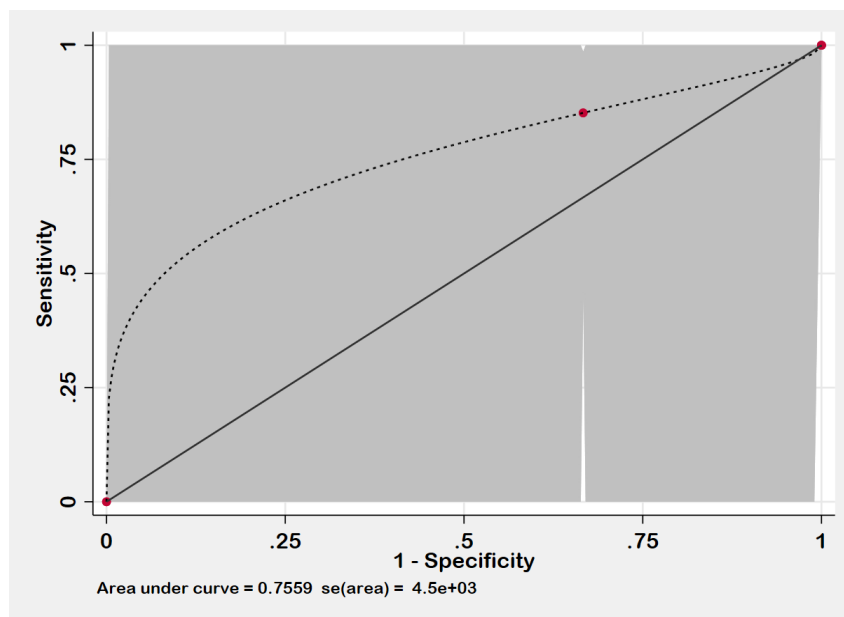


Figure 6. Receiver Operating Curve for IHC Anti E6 Oncoprotein. The area under the curve is 0.9707, which represents 90% chances that the model is able to distinguish between the histopathology and IHC Anti E6 oncoprotein

potentially detectable precursor lesions.

Although early detection of OPMD and OSCC is a desirable goal, evidence supporting the screening is limited, because the progression of oral lesions to cancer cannot be predicted. Dysplasia or even early cancer may be resolved without treatment, which complicates diagnosis and treatment decisions (Epstein et al., 2012). A focus on high-risk populations where prevalence is greater may increase the potential value of screening. The complications regarding screening for low-prevalence diseases lead to challenges in detection and an increased risk of false-positive and false-negative outcomes and higher costs. These challenges continue to challenge oral cancer detection. The current best evidence is limited to high-risk populations, such as those with prior upper aerodigestive tract cancer, exposure to heavy tobacco and alcohol use, exposure to HPV, and immunosuppression. The prevalence of HPV in oral epithelial dysplasia and its association to advance a risk prediction model for the malignant progression of oral epithelial dysplasia can provide further insight into the risk of stratification of oral potentially malignant disorders (Liu et al., 2021).

Associating HPV and head and neck cancer was first mentioned in 1983 by Syrjänen et al., which was then supported by several pieces of evidence like (i) The

epitheliotropic nature of HPV (ii) the confirmed oncogenic potential, seen especially in the cervical squamous cell carcinoma, and (iii) the morphological similarities between oropharyngeal and genital epithelia (Termine et al., 2008). The prevalence of HPV in OSCC is now considered as high as 50% (Mathur et al., 2020).

In order to validate the prevalence of HPV in oral epithelial dysplasia and its association to developing a risk prediction model for the malignant progression of oral epithelial dysplasia, this study aimed to determine whether the repeated measurements of clinical features of OPMDs (lesion presence, size, appearance, color, texture, and histopathology) predict malignant progression. In addition, we tried to detect the immunocytochemistry of HPV E6 and p16 as a valuable test for the detection of high-grade oral epithelial dysplasia compared to using HPV DNA detection without losing sensitivity.

As a cost-effective test, p16 IHC testing has a high sensitivity for HPV (94%) and it is easily applicable on different samples. Although this method is widely recognized, some studies have reported about 20% of p16-positive OPSCCs were HPV-negative, raising questions on the etiological role of HPV in the carcinogenic process (Lewis, 2012). Immunohistochemical evaluation of oral premalignant and malignant lesions for p16

Table 4. Prediction of High-Risk HPV Presence Using p16 and Anti E6 Expression

Statistics	p16		Anti – E6	
	Value	95% CI	Value	95% CI
Sensitivity	18.18%	2.28% to 51.78%	90.91%	58.72% to 99.77%
Specificity	80.00%	56.34% to 94.27%	94.74%	73.97% to 99.87%
Disease prevalence	35.48%	19.23% to 54.63%	36.67%	19.93% to 56.14%
Positive predictive value	33.33%	9.77% to 69.77%	90.91%	59.53% to 98.55%
Negative predictive value	64.00%	55.50% to 71.70%	94.74%	73.47% to 99.15%
Accuracy	58.06%	39.08% to 75.45%	93.33%	77.93% to 99.18%

expression has yielded diverse results with some studies showing reduced expression (Mortazavi et al., 2019; Scully et al., 2007) and others showing increased expression. In our study, only three cases of epithelial dysplasia expressed p16 and were limited to the basal and suprabasal layer. This finding was similar to that of Bradley et al., (2006) indicating heterogeneous expression of p16 within morphologically homogenous tissue. Thus, p16 cannot be a reliable way to differentiate between normal and dysplastic mucosa. Lewis et al., (2012) gave an alternative explanation to the reduced expression of p16 stating that epigenetic mechanisms such as aberrant methylation of p16, DAPK, and MGMT genes could play a role in the progression of premalignant lesions to cancer.

Several studies have shown that HPV status defined by only p16 IHC may be too unspecific. Tagle et al., (2014) analyzed the expression of E6, p53, and p21 proteins and the physical state of HPV16 in cervical cytology. They reported that an elevated E6 expression is an early event of cervical carcinogenesis due to the viral integration into the host genome. A similar trend of stronger E6 expression in the moderately and severely dysplasia was observed in our study.

While analyzing the diagnostic sensitivity and specificity of different tests, the present study revealed positive staining for p16 in 03/30 and 11/30 cases of anti-E6 oncoprotein. Overall, histologic features along with the p16 in all the groups were 33.3% sensitive and 47.1% specific for detecting high-risk HPV with a positive predictive value of 66.67% and negative predictive value of 85.19%. These findings are consistent with the results obtained by Zhu et al., (2019), showing p16-positive cells in cytology preparations requires additional morphologic evaluation to achieve adequate specificity, for which co-expression of p16 and Ki67 seemed to have a higher diagnostic accuracy than p16 alone.

Likewise, histologic features along with the Anti- E6 oncoprotein expressed 90.91% sensitivity and 94.74% specificity with a positive predictive value of 36.67% and negative predictive value of 90.91%. Therefore, Anti E6 expression could serve as a molecular driver of malignancy and might be used as a dynamic indicator in OPMD carcinogenesis.

In conclusion, A major barrier to oral cancer prevention is its failure to predict the risk of development of cancer from precancerous lesions. Our study showed a strong association between HPV and OPMD and summarized the risk prediction by studying different connections between microscopic diagnosis, molecular features, risk habits, clinical lesion characteristics, and their ability to progress into cancer. Secondly, HPV E6 expression could be a valuable test with higher specificity for the detection of high-grade oral epithelial dysplasia compared to HPV DNA detection without losing sensitivity. Solitary P16 expression is insufficient for HPV status detection in OPSCC patients with tobacco and/or alcohol exposure; still, it has better performance in cases without exposure. In conclusion, high expression of Anti E6 oncoprotein in the high-grade stage of dysplasia indicated that lesions might have an increased tendency toward malignancy. Further investigations are needed to provide a better

understanding of the biological behaviour of OSCC caused by HPV.

Author Contribution Statement

Conceptualization and Methodology: Gowthami Jawahar, Gururaj N; Review, writing and editing: Beryl Rachel. J, J. Angeline, B.Nandhinipriya, Swetha. S

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This study is the part of an approved student thesis.

Intuitional Ethical Committee

CSICDSR/IEC/0057/2018

Any conflict of interest

The authors declare that there is no conflict of interests

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