Original Article

A study on *H-score* threshold for p16ink4a immunoperoxidase expression in squamous cell tumours of oral cavity

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Abstract

Background: Validity of various detection methods used are likely contributing factor to this wide variation of prevalence of HPV (0-73%) by using GP5/GP6/MY09/MY11 (L1) primer. PCR is a sensitive method but does not identify transcriptionally active High-risk Human papillomavirus and also does not indicate whether the virus is isolated from malignant tumour cells and non-neoplastic cells. P16ink4a Immunohistochemistry is a highly sensitive and Cost-effective surrogate marker for transcriptionally active high-risk HPV for oral cancer. Objective The aim of the present study was to evaluate the H-SCORE of p16 expression in the surface epithelial tumour sites of a large cohort of squamous cell carcinoma (SCC), severe dysplasia (SD). we sought to determine whether the p16 algorithm is reliable in Oral cavity SCC and severe dysplasia (SD).

Materials and Methods: This study used Immunohistochemistry in archival Formalin-fixed paraffin embedded specimens for assessment of p16 protein expression, cytoplasmic and nuclear staining intensity was categorized based on score (range, 0-3) and presence of tumour cell staining (0-100%).

Results: The majority of positive cases had low H-score of p16 staining except 3/161 (1.8%) cases of tongue SCC had positive for p16 with diffuse moderate staining with ≥2 scores. There were no significant differences in the distribution of demographic, exposure and histopathological characteristics between patients with and without P16 expression.

Conclusion: The present study demonstrated that p16 expression is a reliable HPV marker in the lateral border of the tongue with tonsil involvement but no other sites of the oral cavity. Further p16 IHC detection is required in large cohort of all sites of tongue squamous cell carcinoma studies to validate the marker of HPV.

Keywords: Histopathologic, immunohistochemistry, oral cavity squamous cell carcinoma

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INTRODUCTION

In the incidence of oral cavity cancer cases among men, nearly 104,661 and 31,268 women are diagnosed each year, accounting for oral cancer-related deaths among men 57,216 and among women 18,074 occur yearly in India.[1] Squamous cell carcinoma (SCC) is the most common

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malignancy in head and neck region.[2,3] High-risk human papillomavirus (HR-HPV) has been proven as etiologic agent in subset of the oropharynx (tonsil and tongue base) and this subset is clinically and biologically distinct.[4] High-risk genotypes 16 and 18 are the most commonly detected and transcriptionally active cases reported in 45.8% of oropharyngeal cancers and 24.2% of oral cavity cancers using real-time polymerase chain reaction (PCR).[5]

Though the role of HPV has been established in the etiopathogenesis of oral cavity cancer and its significance of clinical behaviour and outcome is known, there are challenges in the detection of HPV in the clinical setting.[6-11] The validity of various detection methods used is likely a contributing factor to this wide variation. PCR is a sensitive method but may not be feasible in the clinical laboratory setting and often does not necessarily identify transcriptionally active HR-HPV.[12,13] It does not indicate whether the virus is isolated from malignant tumour cells or adjacent non-neoplastic tissues. In-situ-hybridization (ISH) provides evidence of viral localization in the tumour cells but ISH has less sensitivity (88%) and is more expensive compared with immunohistochemistry (IHC) which had a higher sensitivity (96.8%).[12,14] Various studies reported a strong correlation between HPV DNA detection by PCR and P16 IHC. However, the rate of false-positive p16 IHC results ranged from 4% to 38% and this is attributed to various pre-analytical factors such as fixation time, quality of tissue processing, deparaffinization, various staining due to various clones of antibody and dilution used, and lack of strict interpretation criteria for quantification of results such as *H*-score, which is a product of intensity score (0–3) and percentage of tumour staining cell (0–100%).[12,15,16]

In cancer samples, HPV can be found in episomal form and integrated into host cell genome or mixed forms against E6 and E7 oncogene, which decreases the levels of p53 and retinoblastoma tumour suppressor protein, leading to overexpression of p16ink4a IHC.[17] It is most widely used as a surrogate maker for transcriptionally active HR-HPV virus infection in reticulated crypt epithelium in the tonsils and base of the tongue is believed to be the site for HPV-induced carcinogenesis.[18-20] The significance of transcriptionally active HPV infection in oral cavity squamous epithelial malignant cells is unknown at present.[21,22] Cut off points for the p16 H score of malignant cells are not defined in many studies, but AJCC 8TH Manual 2017 has considered that oropharyngeal HPV-induced carcinogenesis is strong, diffuse nuclear and cytoplasmic (>70%) tumour expression with moderate score (2+/3+) of intensity. E6H4 clone in large studies, where patients population with high HPV Incidence, 50% and any staining cut off may be more effective particularly for G175 405 Monoclonal antibody, where HPV incidence is very low.[23,24]

The aim of the present study was to evaluate the *H*-score of p16 expression in surface epithelial tumour sites of a large cohort of SCC and severe dysplasia (SD). We sought to determine whether p16 algorithm reliable in oral cavity squamous cell carcinoma (OCSCC) and SD among patient attending tertiary care hospital in south India by using cut-off threshold 50% based on Jorden *et al*.[25] in their work of Clone G175-104 monoclonal antibody.[6,7]

MATERIALS AND METHODS

This cross-sectional study was approved by the Research Ethics Committee in human studies of JIPMER, Puducherry, India. The inclusion criteria were patients' histopathological confirmed diagnosis of oral squamous cell carcinoma (OSCC) and SD patients who agreed to participate in the study. The histopathological diagnosis of each case was confirmed and histological grade was determined by independent oral pathologist. Demographic, exposure and clinical data information recorded at enrolment. A total of 161 formalin-fixed, paraffin-embedded (FFPE) biopsy block retrieved from the Pathology Department.

Immunohistochemical analysis

FFPE tissue sectioned into slices as thin as 4–5 μm with a microtome and mounted onto positively charged microscopic slides (Biogenex optiplus™ CA USA). After mounting, the sections were dried in an incubator at 60°C for 1 h and then 37°C for overnight. Deparaffinize the slide in two changes of xylene for 10 min each with agitation, followed by rehydrated in two changes of ethanol for 10 min.

The slides were placed in distilled water for 5–10 min and incubated slides with hydrogen peroxide (0.3–12% v/v) in methanol for 20 min to quinch the endogenous peroxidase activity; rinse slides with Tris-buffered saline (TBS, 0.1 M, pH 7.4) three changes were being used. Antigenic recovery was performed with citrate buffer with six whistles by pressure cooker method (one whistle at high temperature and five whistles at medium temperature); slides were rinsed with distilled water once and then with wash buffer (TBS for 5 min). Secondary blocking was done after antigen retrieval. Remove the slide from the copulin jar and carefully wipe the edges and place the slide in IHC staining box horizontally. Cover the section with 200 μl 3% BSA on each section and incubate at room temperature for 20 min. Sections were incubated with p16Ink4a monoclinal

antibody (prediluted MAB, Biogenex, USA) for 16 h at 4°C temperature and washed with three changes of buffer. HRP was used as the detection system for 40 min and washed with three changes of TBS buffer. Incubated all slides with chromogenic substrate-diaminobenzidine (liquid DAB) in order to develop brown colour in sections and washed in distilled water and Mayer's haematoxylin was used as anti-staining. Positive control of known p16-positive endometrial carcinoma was run with each batch of test slides. A negative control, wherein the primary antibody was omitted, was also put with each batch of the test slides to maintain the validity of the immunostaining procedure [Figure 1].

Evaluation of immunostaining and scoring

Tumour p16 expression was evaluated by immunohistochemical analysis with mouse monoclonal antibody anti-p16INK4a (G-175-104 clone) against protein p16INK4a, in FFPE tissue under a microscope using 100 and 400× original magnification. Representative image of p16 slide was digitally scanned on an Aperio image scanner at 10× and 40× magnification. For assessment of p16 protein expression, cytoplasmic and nuclear staining intensity was categorized based on score (range, 0–3) and presence of tumour cell staining (0–100%).[8] Fifty per cent cut-off threshold with 2+/3+ intensity score selected for p16-positive cases based on previously suggested work by Jorden *et al*.[25] P^{16} IHC was also reviewed by two additional pathologists (NG and SC) in order to assess the inter-observer variability.

OBSERVATION AND RESULTS

One-hundred and sixty-one patients, newly diagnosed by independent pathologist, were enrolled in the present immunohistochemical study. The clinico-epidemiological and pathological characteristics of OSCC, verrucous squamous cell carcinoma (VSCC) and SD are documented in Table 1, which summarizes demographic, exposure and clinical characteristics of patient with and without p16 expression. Among the 161 tumours, p16 expression was evaluated by immunohistochemical analysis with mouse monoclonal antibody anti-p16INK4a (G-175) against protein p16INK4a, in 4–5 μm section of formalin-fixed, paraffin-embedded (FFPE) tissue. Of these 14 (8.7%) patients, positive staining for p16 expression was found in the sample of buccal mucosa 5/161 (3.1%), followed by tongue 5/161 (3.1%), vestibule of mouth 2 (1.2%), gingivobuccal sulcus and hard palate (0.6%), but there was no significant association between p16 and sites of oral cavity [Table 2]. The majority of positive cases had low *H*-score of p16 staining except 3/161 (1.8%) cases of tongue SCC had positive for p16 with diffuse moderate staining with ≥2 score [Figure 2]. With the 50% cut-off score, there was perfect agreement (100%) and kappa value (1.00) among three pathologists. There were no significant differences in the distribution of demographic, exposure and histopathological characteristics between patients with and without P16 expression.

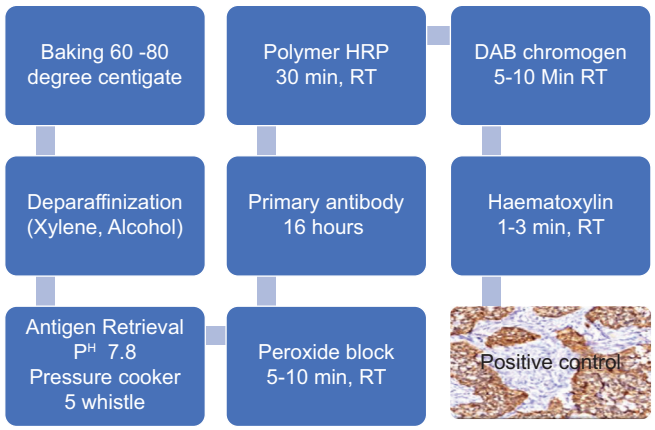

Figure 1: Immunohistochemistry-standardized protocol for p16ink4a marker (clone G175-104 Mouse Monoclonal, Biogenix, CA) in formalin-fixed, paraffin-embedded tissue sections of oral epithelial lesions. Washing steps in between by using Tris-buffered saline (pH 7.6) and distilled water

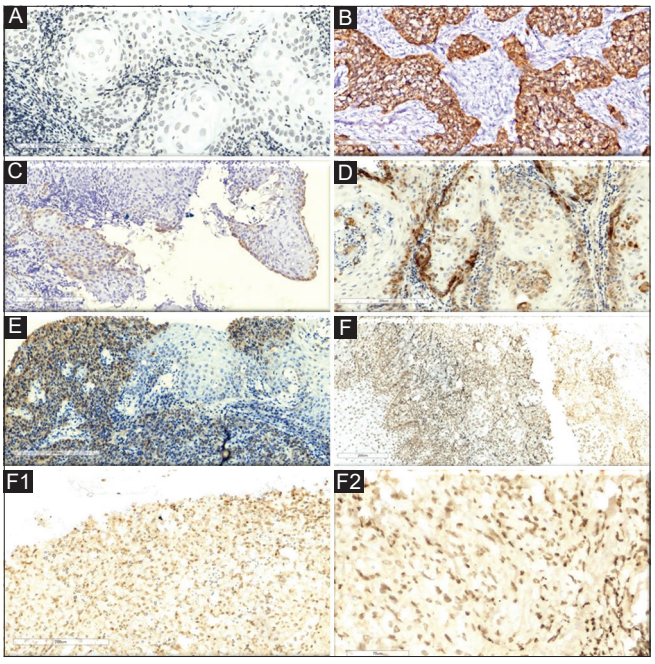

Figure 2: Representative image of $p16^{ink4a}$ immunohistochemical finding of clone G175-104 MAB. Negative control (A), positive control (B, cervical SCC; 100×), mild staining of cytoplasmic (C); moderate intensity cytoplasmic and nuclear staining in $p16^{ink4a}$ negative cases (score 0–2, ≤50% of tumour cell). Moderate and patchy cytoplasmic and nuclear immunostaining is present (score >2; ≥50% of tumour cell) in p16ink4a-positive cases; (100× D& E). Mild and diffuse p16 nuclear and cytoplasmic staining present ≥50% tumour cell in p16-positive cases (F–F1–F2)

Table 1: p16^{ink4a} status by immunohistochemistry (IHC) and demographic and clinical characteristics in patients with squamous cell carcinoma and severe dysplasia of oral cavity

Variables	Factor	P16 Negative	P16 positive	Total	*P*
Gender	Male	101 (94.3)	6 (5.6)	107	0.073
	Female	46 (85.1)	8 (14.8%)	54	
Age (years)	≤50	46 (93.9)	3 (6.1)	49	0.55
	≥50	101 (90.2)	11 (9.8)	112	
Tobacco history	Never	68 (93.2)	5 (6.8)	73	0.57
	Ever	79 (89.8)	9 (10.2)	88	
Alcohol history	Never	89 (91.8)	8 (8.2)	97	0.7
	Ever	58 (90.6)	6 (9.4)	64	
Smoking history	Never	94 (91.3)	9 (8.7)	103	0.98
	Ever	53 (91.4)	5 (8.6)	58	
Diet history	Vegetarian	58 (89.2)	7 (10.7)	65	0.57
	Non-vegetarian	89 (92.7)	7 (7.3)	96	
Degree of keratinization	Keratinized	63 (90)	7 (10)	70	0.77
	Non-keratinized	84 (92.7)	7 (7.7)	91	
Histologic grade	WDSCC*	93 (92.1)	8 (7.9)	101	.062
	MDSCC*	31 (88.6)	4 (11.4)	35	
	PDSCC*	0 (0)	1 (100)	1	
	VSCC*	5 (83.3)	1 (16.7)	6	
	SD±	18 (100)	0 (0)	18	

Ever smoker: Who had smoked at least 100 cigarettes in their life time. Ever drinker: Who had drunk at least one alcoholic beverage/week for at least 1 year. Ever user tobacco: Who had daily tobacco use for at least 1 year. *Squamous cell carcinoma, ±severe dysplasia

Table 2: Association between the anatomic sites and p16^{ink4a} expression in oral epithelial tumour of oral cavity

ICD-O*	P16		Total	*P*
	Negative	Positive		
C0.0.4	1	0	1	0.966
C0.2.0	15	2	17	
C0.2.1	18	3	21	
C0.3.1	9	1	10	
C0.4.9	9	0	9	
C0.5.0	8	0	8	
C0.6.0	57	7	64	
C0.6.1	19	1	20	
C0.6.2	5	0	5	
C0.6.8	6	0	6	
Total	147	14	161	

*International classification of diseases: oncology

DISCUSSION

High-risk HPV infection in sites outside the oropharynx and the methods of choice to diagnose infection have not clearly defined but some studies expressed p16 IHC marker as surrogate marker for HPV infection in the head and neck sites, even those outside the oropharynx.[9,10] P16 IHC is currently used as a marker for detecting transcriptionally active HPV in oropharyngeal SCC.[11,12] In the present study, p16 expression has studied various anatomical sites of oral cavity and grade of SCC and dysplasia. The majority of positive cases had low *H*-score of p16 staining except 3/161 (1.8%) cases of tongue SCC had positive for p16 with diffuse moderate staining with ≥2 score. Zero prevalence of HPV and p16 transcriptionally active HPV cases was found leukoplakia (0/121) and oral cavity (0/296) in Bhosale studies.[13] Studies reported cut-off criteria for positivity >25%, 50% and 70% for G175-104 and E6H4 clone.[14,15] New evidence-based guideline for HPV testing of head and neck cancer showed that p16 is reliable marker for oropharyngeal SCC if cut-off criteria for p16 are more than 70% with diffuse strong continuous cytoplasmic and nuclear staining; so other reasons for false-positive staining especially in small biopsies would be eliminated.[16] In systematic review by Kreimer *et al*.,[26] HPV DNA was detected in 2.4% of OCSCC worldwide. In the present study, p16 positive was detected in 2/161 (1.2%) if cut-off threshold has taken ≥2 score (51–100% tumour cell staining). There was high variability of HPV positivity in oral epithelial dysplasia (0–80%) which was reported by various investigators, whereas some of the studies have not defined the *H*-score criteria for P16.[17,18] This study showed no p16 positivity in oral SD based on cut-off score. Our results are consistent with the previous studies of OCSCC showing range 5.4–62%).[19-21]

CONCLUSION

This study demonstrated that p16 overexpression is reliable HPV marker in tongue SCC based on cut-off threshold 50%, but there are no other sites of oral cavity. Further, p16 IHC detection is required in a large cohort of all sites of tongue SCC studies with ≥50–100% tumour cells positive for staining criteria for G175-104 clone to validate the marker of HPV.

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Conflicts of interest

There are no conflicts of interest.

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