WILEY Journal of Oral Pathology & Medicine

The role of human papillomavirus in p16-positive oral cancers

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Background: The aim of this study was to identify the presence and frequency of human papillomavirus (HPV) nucleic acid in p16-positive oral squamous cell carcinomas (OSCCs), to assess whether the virus was transcriptionally active and to assess the utility of p16 overexpression as a surrogate marker for HPV in OSCC.

Methods: Forty-six OSCC patients treated between 2007 and 2011 with available formalin-fixed paraffin-embedded (FFPE) specimens were included. Twenty-three patients were positive for p16 by immunohistochemistry (IHC) and these were matched with 23 patients with p16-negative tumours. Laser capture microdissection of the FFPE OSCC tissues was undertaken to isolate invasive tumour tissue. DNA was extracted and tested for high-risk HPV types using a PCR-ELISA method based on the L1 SPF10 consensus primers, and a real-time PCR method targeting HPV-16 and HPV-18 E6 region. Genotyping of HPV-positive cases was performed using a reverse line blot hybridization assay (Inno-LiPA). RNAScope® (a chromogenic RNA in situ hybridization assay) was utilized to detect E6/E7 mRNA of known high-risk HPV types for detection of transcriptionally active virus.

Results: HPV DNA was found in 3 OSCC cases, all of which were p16 IHC-positive. Two cases were genotyped as HPV-16 and one as HPV-33. Only one of the HPV-16 cases was confirmed to harbour transcriptionally active virus via HPV RNA ISH.

Conclusion: We have shown that the presence of transcriptionally active HPV rarely occurs in OSCC and that p16 is not an appropriate surrogate marker for HPV in OSCC cases. We propose that non-viral mechanisms are responsible for the majority of IHC p16 overexpression in OSCC.

KEYWORDS
head and neck cancer, human papilloma virus, oral cancer, oral squamous cell carcinoma, p16

1 | INTRODUCTION

Head and neck squamous cell carcinomas (HNSCC) are the sixth most common cancer worldwide.[1] Tobacco and alcohol use are the principal risk factors. However, 25%-35% have been shown to be associated with human papillomavirus (HPV), and these are mostly confined to the tonsil and base of tongue.[2,3] Patients with HNSCC HPV-positive oropharyngeal tumours have distinct clinical features

and a more favourable prognosis[3] compared to HPV-negative HNSCC.

The oral cavity is the most common non-oropharyngeal HNSCC site where HPV is implicated.[4] Nevertheless, the role of HPV in oral cancer is controversial, with reported prevalence of HPV ranging from 0% to 90%.[2,5-7] Some studies may have mixed oral cavity and base of tongue tumours, leading to falsely higher rates of HPV in oral cancer. At present, the prevalence and role of HPV in non-oro-pharyngeal sites remains unclear and a causal relationship has not been established. Immunohistochemical (IHC) staining for p16 is used as a surrogate marker for HPV infection in oropharyngeal SCC, with HPV-positive tumours overexpressing p16.[8]

HPV oncogenes E6 and E7 are required for tumour initiation and immortalization.[9] They are highly associated with carcinogenetic activity and are responsible for inhibition of the tumour suppressors p53 and retinoblastoma protein (pRb).[10,11] Moreover, E7-driven inactivation of pRb in high-risk (HR) HPVs has been shown to lead to p16 overexpression.[3,12] This interferes with cell cycle control and promotes genetic instability and cancer progression.[11,13]

In a previous study,[14] we performed retrospective immunohisto-chemical (IHC) staining to determine the expression of several proteins in 129 formalin-fixed paraffin-embedded (FFPE) specimens from a well-defined cohort of consecutive oral squamous cell carcinoma (OSCC) patients. We found 23 (18%) of these patients displayed p16 overexpression, which was significantly increased in non-smokers and non-drinkers who were less than 70 years old. This result warranted further investigation given the association between p16 overexpression and HPV in HNSCC. We hypothesize that if p16 could be used as a surrogate marker for HPV in OSCC, it may further indicate prognosis.

Our first aim for this study was to determine whether p16 IHC-positive OSCCs actually harbour HPV. Secondly, we aimed to identify either HPV E6 or E7 mRNA in these tumours as a marker of transcriptionally active virus that may be responsible for oncogenesis in these patients. Finally, we aimed to assess the utility of p16 as a surrogate marker for HPV infection in OSCC.

2 | MATERIALS AND METHODS

2.1 | Patients

Twenty-three OSCC p16 IHC-positive patients were matched to a control cohort of 23 OSCC p16 IHC-negative patients. Matching was performed using semi-parametric and nonparametric matching methods as implemented in the MatchIt R package.[15] Matching was attempted on age, gender, T stage (divided into early [T1-T2] and late [T3-T4] stages), N stage (divided into node-positive and node-negative), tobacco use (which included those with >5 pack years) and alcohol use (which included <3 drinks per week). Exact matches were found for gender, T stage, N stage and tobacco use, with a similar mean age between the two groups. Archived FFPE sections were obtained for each of the 46 patients. All tumours were in oral cavity sites only. Mortality data were sourced from both the ACCORD (the

Australian Comprehensive Cancer Outcomes and Research Database) and the Victorian Cancer Registry databases (census date 31 December 2015). Expression levels of p16 were determined by IHC staining using p16 mouse monoclonal antibody (Roche, Basel, Switzerland). The staining was scored as previously described.[14]

This study was approved by the Melbourne Health Human Research Ethics Committee 19 September 2012 (MH Project number 2012.071).

2.2 | Histological preparation of samples

Histological sectioning of FFPE tissue blocks was performed by conventional sterile technique at the laboratory service of the Anatomical Pathology Department, within the Royal Melbourne Hospital, Melbourne, Australia. To minimize cross-contamination between sectioning of each tissue sample, the microtome blade was changed and the microtome surface cleaned with xylene. Each section was mounted on superfrost plus slides. Haematoxylin and eosin (H&E)-stained sections were reviewed and annotated by a consultant pathologist to define tumour-containing regions.

2.3 | Laser capture microdissection (LCM) and HPV DNA Detection

HPV DNA detection was performed in the Western Pacific Regional HPV Labnet Reference Laboratory within the Molecular Microbiology Department of the Royal Women's Hospital, Melbourne, Australia; 4-μm-thick mounted FFPE sections were deparaffinized via sequential 5-minute incubations in 100% xylene (twice) and 100% ethanol (twice). The slides were then incubated at 37°C degrees for one hour. Following this, the annotated H&E-stained tissue section was used to guide capture of the tumour area of interest on the Veritas 704 Laser Capture Microdissecting System (Arcturus Bioscience, Mountain View, CA, USA). Cells were captured onto a thermoplastic CapSure Macro LCM Cap (Applied Biosystems, Foster City, CA, USA) over the target tissue by pulsing with an infrared laser to adhere the cells to the cap. DNA was extracted from each cap with the Arcturus PicoPure DNA Extraction Kit (Applied Biosystems) as per protocol F of the PicoPure DNA Extraction Kit user guide. DNA from LCM-extracted tissue sections was first tested for extraction efficiency by assessing the presence of intact endogenous human genomic DNA. This was established by performing a 110-base pair (bp) beta-globin real-time PCR assay using the LightCycler® 480 Instrument II (Roche) as previously described.[16] All PCR conditions are outlined in Table 1.

LCM-extracted DNA was then tested for HPV DNA on the DNA ELISA kit HPV SPF10, V.1 (Labo Bio-medical Products BV, Rijswijk, the Netherlands), which is validated for detection of 44 HPV genotypes, according to the manufacturer's instructions. Samples that were positive for HPV DNA on the DNA ELISA were further tested for HPV genotype on the RHA kit HPV SPF10-LiPA25, V.1 (Labo Bio-medical Products BV). Hybridization of the biotinylated amplicons to the detection strip was performed using the Auto-LiPA 30 instrument (Innogenetics) according to the manufacturer's

TABLE 1 Thermocycler conditions for each PCR target

Target	Initial denaturation	Amplification	Number of cycles	Cooling
Beta-globin	95°C for 5 min	95°C for 10 s, 60°C for 30 s, 72°C for 1 s	45	40°C for 1 min
L1-SPF10	94°C for 9 min	95°C for 30 s, 52°C for 45 s, 72°C for 45 s	40	72°C for 5 min
HPV-16/18 E6	95°C for 10 min	95°C for 10 s, 55°C for 10 s, 65°C for 20 s	55	40°C for 1 min

instructions.[17] SPF10-LiPA allows for identification of 25 different HPV genotypes, which include 15 high-risk types (HPV-16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68 and 73) and 8 low-risk types (HPV-6, 11, 34, 40, 43, 44, 54, 70 and 74). Additional screening of all samples for HPV-16 and HPV-18 E6 gene via real-time PCR assay was performed using the LightCycler® 480 Instrument II (Roche) as previously described,[16] targeting a 102-bp fragment of the HPV-16 E6 and a 142-bp fragment of the HPV-18 E6 regions.

2.4 | HPV RNA detection

RNA in situ hybridization (ISH) for detection of high-risk HPV E6/E7 mRNA was performed in the Translational Research Laboratory within the Peter MacCallum Cancer Centre of the Victorian Comprehensive Cancer Centre, Melbourne, Australia, using the RNAScope® HPV kit (Advanced Cell Diagnostics, Hayward, CA, USA) according to the manufacturer's instructions. RNA ISH was performed on all cases in which HPV DNA was detected by PCR (n = 3) to assess for the presence of transcriptionally active virus, and also as an extra test on all cases where the 110-bp beta-globin was not detected (n = 10). One FFPE section from each case was hybridized with a single cocktail target HRP-labelled probe of 18 high-risk (HR) HPV genotypes (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82). A second FFPE section from each case was used as a positive control and hybridized with probes to endogenous peptidyl-prolyl cis-trans isomerase B (PPIB) mRNA, to demonstrate detectible mRNA in the FFPE samples. Specific staining signals when present were identified as brown, punctate dots located in the cytoplasm and/or nucleus and qualitatively classified in a binary manner as either positive (present) or negative (absent). Only cases showing positive control probe signals were scored for the presence or absence of HPV probe signals.

Following RNAScope® ISH, seven cases remained inconclusive as testing did not detect either of the internal controls, 110-bp beta-globin DNA target on LCM-extracted DNA or PPIB mRNA target on ISH. Subsequently, a shorter fragment (70 bp) human genomic beta-globin qPCR was designed and validated (validation data not shown). The assay comprised of PC03_4 TaqMan probe and PCO3 forward primer, with a new reverse primer, PCO5 (5′- GTGCATCTGACTCCT GAGGA-3′). The 70-bp beta-globin qPCR was performed on the LCM-extracted DNA from all inconclusive cases to confirm the presence of endogenous human genomic DNA.

2.5 | Statistical analysis

Statistical analysis was performed using the R software package (R Foundation for Statistical Computing, Vienna, Austria). The 5-year survival rate was analysed using the Kaplan-Meier method. Hazard ratios were obtained using the univariate Cox proportional hazards model. Statistical significance was set at $P \leq .05$.

3 | RESULTS

The study population consisted of 23 patients with p16-positive OSCC and a further 23 matched patients with p16-negative OSCC. Of the 46 patients, 33 (72%) were under 70 years of age and were mainly smokers (32 patients, 70%) or alcohol drinkers (29 patients, 63%). Tumour sites were spread across the oral cavity, with the highest proportion (43%) located in the oral tongue (anterior two-thirds of the tongue). Thirty per cent of cancers were stage I. At 5 years, 28% of patients had died from their disease (Table 2). p16 expression was not found to be associated with 5-year disease-specific survival ($P = .694$ by log rank test) (Figure 1). However, p16-positive patients were found to have a lower risk of death from OSCC than p16-negative patients (HR = 0.47; 95% CI, 0.14-1.55).

HPV L1 DNA was found by SPF10-LiPA PCR assay in 3 male smoking and drinking patients who were all p16-positive. All 3 patients were alive at the census date. Genotyping identified 2 cases as HPV-16 and the remaining case as HPV-33. The HPV-33 and one of the HPV-16 cases were strongly positive by SPF10-LiPA assay with the optical density of their PCR products being four times greater than the HPV-positive cut-off value on ELISA. This resulted in a dark hybridization band on LiPA. The other HPV-16 case was borderline positive, with the optical density of the PCR product at the HPV-positive cut-off value on ELISA resulting in a lighter band on LiPA (Figure 2). HPV-16 E6 DNA was also identified in the case that was strongly positive for HPV-16 L1 DNA. All other samples were HPV-16/HPV-18 E6 DNA-negative. Housekeeping genes could not be detected in 2 of the 46 samples and these were excluded from further analysis, as they could not definitively be considered as HPV-negative.

RNAScope® ISH was performed for the 13 samples which were either HPV DNA-positive by PCR (as a confirmatory test for transcriptionally active virus) or 110-bp beta-globin-negative (as an alternative test). Eight of these samples were p16-positive and 5 were p16-negative. Housekeeping RNA was detected in 6 samples, but was most likely degraded in the 7 samples in which the housekeeping/control mRNA probe was undetectable, including the HPV-33-positive sample. Only 1 of the 3 HPV DNA PCR-positive samples (which was both HPV-16 L1- and HPV-16 E6-positive by PCR) was also positive for the high-risk HPV mRNA RNAScope® ISH probe (Figure 3). This mRNA was not identified in the other HPV-16 L1-

TABLE 2 Clinicopathological features of our patient cohort

	p16 IHC-positive	p16 IHC-negative	Total	%
Gender				
Male	13	13	26	57
Female	10	10	20	43
Age				
<70	17	16	33	72
>70	6	7	13	28
Tobacco				
Smoker	16	16	32	70
Non-smoker	7	7	14	30
Alcohol				
Drinker	13	16	29	63
Non-drinker	10	7	17	37
Stage				
I	6	8	14	30
II	6	5	11	24
III	4	5	9	20
IV	7	5	12	26
Site				
Tongue (anterior one-third)	12	8	20	43
Mandibular Alveolus	1	1	2	4
Maxillary Alveolus	2	5	7	15
Floor of Mouth	1	4	5	11
Hard Palate	1	1	2	4
Cheek Mucosa	3	2	5	11
Vestibule of Mouth	0	0	0	0
Retromolar Region	3	2	5	11
Outcome after 5 years				
Deceased	5	8	13	28
Alive	18	15	33	72

Non-smoking status has been defined as a negligible history of tobacco use and non-drinking status as no regular alcohol consumption or previous history of heavy alcohol intake or abuse. Stage has been classified according the American Joint Committee on Cancer (AJCC) sixth edition system.

positive sample. HPV DNA or mRNA was not detected in any of the p16-negative samples (Table 3).

In summary, of the 44 cases, only one sample was HPV E6/E7 mRNA-positive and three samples were HPV DNA-positive. Within the study period, twelve patients had died of disease and three from other causes. Too few HPV-positive tumours were identified to perform meaningful statistical analysis.

4 | DISCUSSION

Most studies assessing the role of HPV in OSCC utilize DNA detection as an indicator of the presence of HPV. This is the first study, to our

Kaplan-Meier plot
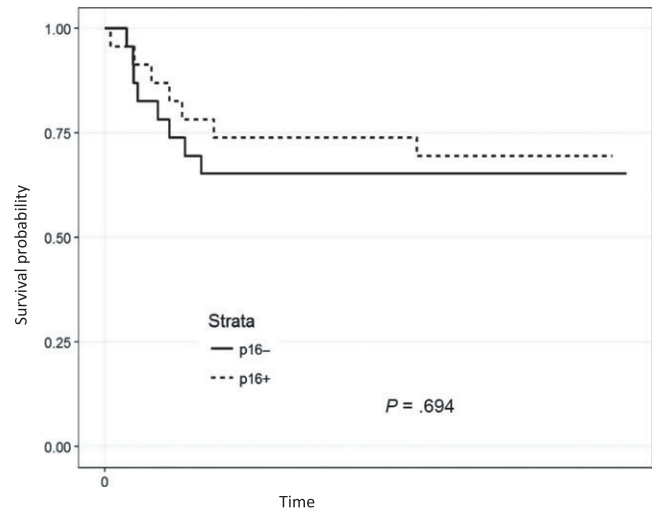
FIGURE 1 Kaplan-Meier survival curve comparing p16-positive and p16-negative populations

knowledge, to examine OSCC for evidence of HPV by two orthogonal methods in archived FFPE tissues: tumour-associated DNA using LCM-PCR and transcriptional activity by mRNA ISH. Using both HPV DNA and RNA detection methods, we have shown that HPV may be a causative factor in OSCC, albeit at low prevalence (1 patient, 2.3%) in our small cohort. Our frequency of HPV-positive tumours, using more precise laboratory methods, is concordant with the existing literature.[13,18] We have shown that p16 overexpression is only rarely HPV-related and p16 IHC expression cannot be used as a surrogate marker for the presence of HPV in oral cavity carcinomas.

It was surprising that so few samples were HPV-positive, with just 3 of 44 cases containing detectable tumour-associated HPV DNA. Two cases harboured HPV-16, and a further case harboured HPV-33. Studies have shown that HPV-16 is the most frequent viral subtype found in HNSCC, followed by HPV-33.[19] However, detection of HPV DNA does not in itself correlate with biological activity and oncogenesis,[20] especially in the absence of E6/E7 transcripts.[21] Only one HPV DNA-positive OSCC in our cohort was also positive for HPV E6/E7 mRNA, genotyped as HPV-16. This is the only case in our cohort we can confidently attribute to HPV. The SPF10-LiPA PCR result in the other HPV-16-positive sample was borderline positive, and this sample was also negative by HPV-16/HPV-18 E6 PCR and RNA ISH. This was not unexpected as the SPF10-LiPA assay has a higher sensitivity due to the shorter amplicons (65 bp) covering the target. This cancer cannot conclusively be considered as HPV-driven, with no evidence of viral transcription. The mRNA control was not detected in the HPV-33 sample, rendering this case inconclusive for transcriptionally active infection.

There is no consensus method to detect HPV in HNSCC, especially for archived FFPE tissues. HPV identification in FFPE tissues is challenging, as both DNA and RNA are degraded by formalin fixation, age and variable storage conditions, which reduces PCR efficiency and assay sensitivity.[22] The analysis of RNA in this archival tissue is particularly challenging as tissue autolysis prior to fixation can result in degradation of RNA of resected tumours, and RNA is
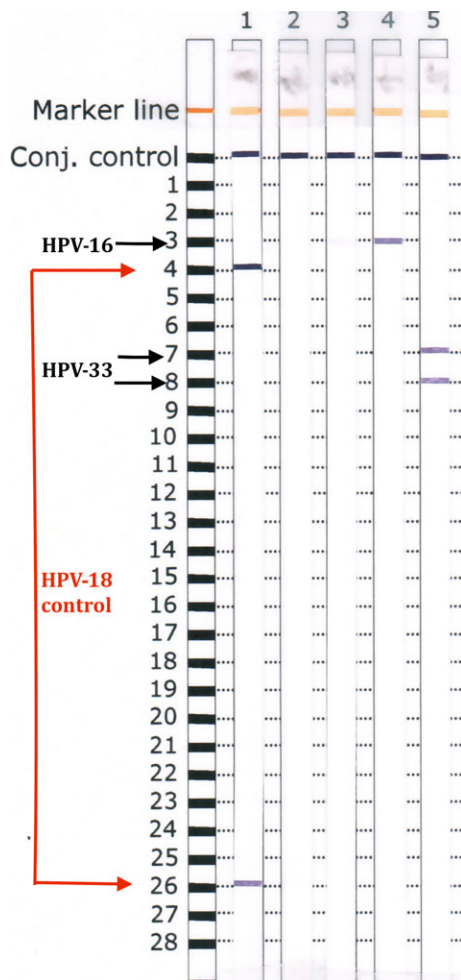
FIGURE 2 INNO-LiPA human papillomavirus (HPV) Genotyping extra nitrocellulose membrane strips in lanes 1-5. Hybridization bands are analysed by comparing the probe number to the standard grid provided by the manufacturer. Combinations of single or multiple probes correspond to specific HPV types. Lane 1 is the positive control, lane 2 is the negative control, and lanes 3-5 are HPV L1 DNA-positive samples

cross-linked to adjacent molecules (DNA and proteins) during formalin fixation[23] and can be further damaged by prolonged storage time and poor storage conditions of paraffin blocks.[24]

The utilization of SPF10 primers for the DNA analysis has been shown to be ideal and have a higher sensitivity for degraded HPV sequences given their shorter amplicon lengths.[17,25] In FFPE, this is superior to other clinical assays available[26] that broadly target the L1 region[17] and enable the amplification of multiple HPV types.[27] SPF10 HPV PCR can be combined with a reverse hybridization line probe assay (LiPA) permitting simultaneous detection of different HPV types individually in a single assay.[25] Additional RT-PCR assays with primers targeting the HPV-16 and HPV-18 E6 region are necessary as HPV L1 regions can be lost following HPV DNA integration into the host genome, leading to false negatives in more advanced disease.[4] The E6 nucleotide sequence exhibits less nucleotide variation,[28] and targeting two different viral gene locations improves sensitivity.

RNA detection was achieved by RNA ISH (RNAScope®) targeting the actively transcribed E6/E7 mRNA region to verify viral integration and subsequent mRNA transcription. This assay would ideally have been performed on the entire patient cohort. However, in preliminary attempts to optimize the assay, the housekeeping/control mRNA probe could not be detected in 20 of 30 cases. This is likely as a result of RNA degradation in our samples, which is unavoidable in FFPE tissues that have been stored for some time. Further analysis was limited by the amount of FFPE material available.

The prognostic significance of p16 IHC overexpression has been attributed to improved outcome in oropharyngeal cancer (OPC) cases.[29] p16 overexpression has been shown to correlate with HPV infection in malignant oral lesions[30] with some authors advocating its use as a surrogate marker. Accurate identification of HPV-driven OPC is still a major issue. Algorithms combining p16 and HPV DNA testing perform better than p16 testing alone in OPC cases[31] and we hypothesized that the same might be true for non-OPC HNSCC such as OSCC. However, we found that whilst all patients in the cohort with detectable HPV were p16 IHC-positive, p16 expression was not a predictor of HPV status in OSCC. We also found no statistically significant association between p16 overexpression and survival in our study cohort, which is similar to studies involving non-OPC patient cohorts,[18,32] but is in contrast to the study by Chung et al.[33]

Whilst our results have shown that there is no proven prognostic difference based on HPV status in this small cohort of OSCC,
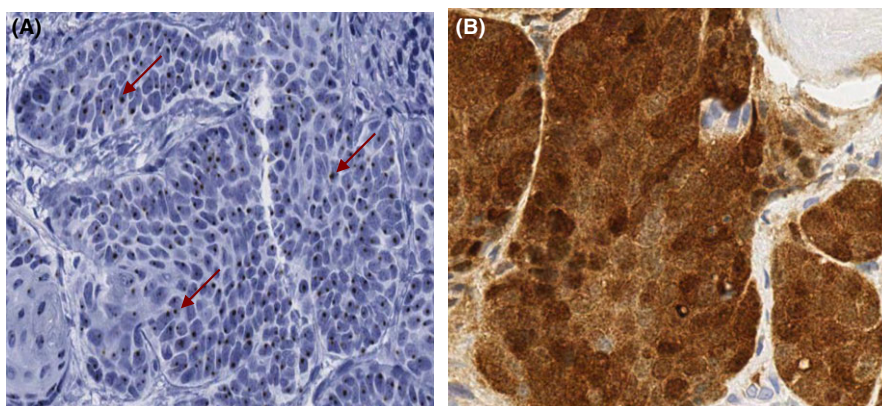
FIGURE 3 A, RNAScope® detection of E6/E7 mRNA transcripts as identified as punctate, brown nuclear staining in tumour cells (arrows showing some examples of nuclear signals). B, p16-positive immunohistochemistry staining. Magnification ×400

TABLE 3 Summary of results from the various methods used to analyse samples

	DNA control (n = 46)		HPV DNA PCR (n = 46)		RNA ISH PPIB Control (n = 13)		HPV mRNA ISH (n = 13)	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
p16 IHC-positive	23	0	3	20	6	2	1	7
p16 IHC-negative	21	2	0	23	0	5	0	5
Total	44	2	3	43	6	7	1	12

"DNA control" includes both 70-bp and 110-bp beta-globin PCR housekeeping gene; "HPV DNA" includes both SPF10-Lipa PCR and HPV-16/18 E6 qPCR. Only one RNA ISH result was mRNA HPV-positive, which was positive to the PPIB control and p16 IHC-positive.

additional HPV-specific tests could be implemented in OSCC cases pending a large-scale cost/benefit analysis. It is hard to justify the implementation of a screening programme from results in the present study on the basis that only one case harboured transcriptionally active HPV. However, if this were to occur, we would recommend that LCM be performed prior to sensitive PCR-based HPV testing, to reduce false-positive results. The oral cavity can be colonized by oncogenic HPV without disease, usually cleared within the first year,[34] and PCR may detect HPV infection in normal epithelium in addition to tumour tissue.[35] LCM prior to DNA extraction precisely selects tumour cells and prevents contamination by adjacent tissue.[36] LCM-PCR has been shown to be the preferred method for HPV detection in cervical neoplasms.[37] Further analysis should be performed in positive samples by RNAScope® ISH to confirm the presence transcriptionally active HPV. This has a potential laboratory turn-around time of 2-3 days.

Recent epidemiological data reported 300 400 new cancers of oral cavity and lip cancers in 2012 globally, which resulted in 145 400 deaths.[38] For over 50% of cases diagnosed annually, oral cancer remains a lethal disease.[1] Treatment might be personalized for the small subgroup of patients with HPV-positive OSCC, with the development of less morbid treatment regimens. From a public health perspective, there are further benefits to a HPV vaccination programme, reducing the prevalence of yet another cancer.

The role of HPV in OSCC is more nuanced than in the oropharynx, with our study suggesting a non-viral cause of p16 overexpression in this group. We do not recommend that p16 IHC alone be used as a surrogate marker for the presence of HPV in OSCC.

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