HPV detection in primary intra-oral squamous cell carcinomas – commensal, aetiological agent or contamination?

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Abstract

Background: High-risk human papilloma viruses (HPV) are reported to be significant independent risk factors for oral squamous cell carcinoma (OSCC). The prevalence of HPV in OSCC in a South African population sample was evaluated comparing three different HPV detection methods.

Methods: Tumour and adjacent morphologically normal oral mucosa of 59 resections of primary OSCC were evaluated for the presence of HPV using real-time polymerase chain reaction (PCR), conventional *in situ* hybridization (ISH), and a signal amplification ISH technique (Dako GenPoint^{TM}).

Results: HPV18 DNA was detected in seven cases using real-time PCR. No positivity was found with the other two ISH techniques.

Conclusions: We support the view that HPV is probably unimportant in the pathogenesis of OSCC and hypothesize HPV detection techniques as the main reason for the positive results in many studies. Real-time PCR was confirmed as the most sensitive technique, but researchers are urged to incorporate strict internal controls when using this detection method.

Introduction

Intra-oral squamous cell carcinoma (OSCC) is the eighth most common malignancy in the developing world (1). High-risk human papilloma viruses (HPV) types 16 and 18 as aetiological agents of anogenital carcinoma have been firmly established in the literature (2). Because of the morphological similarities (3) and epitheliotropic nature of HPV, a link between OSSC and HPV seemed logical and has been the focus of numerous studies. The relationship between HPV and the oral mucosa has been supported by several investigators reporting the presence of HPV-DNA in healthy oral mucosa (4, 5) as well as in OSCC (6). A predilection of HPV for certain, especially non-keratinized anatomical sites in the oropharynx, was confirmed when HPV16-related DNA sequences were detected in 16%, 51%, 60% and 13% of SCC of the tongue, tonsil, Waldeyer's ring, and pharynx respectively (7–9). This resulted in the identification of a distinct subset of head and neck SCC, particularly tonsil carcinoma, which have a strong and consistent association with high-risk HPV types with molecular characteristics indicative of viral oncogene function (10). HPV16 and 18 are the most widely implicated HPV types in the OSCC literature (6). Estimates on

the prevalence of oral HPV in normal mucosa, pre-malignant lesions and OSCC, is however highly variable and comprehensive reviews have reported results that varied from 0% to 100%. These results seemed to depend on the sampling methods, patient profile (11, 12), detection methods used (13), and the anatomical location of the tumours (14).

Cancer of the oral cavity, pharynx, tongue, and gums is one of the most common malignancies registered in the cancer registries of South Africa (15, 16). A retrospective study in 1995 found only one HPV18-positive tumour using *in situ* hybridization (ISH) in 66 cases of OSCC in a rural Black South African population sample (17). The same group studied a different African population sample performing standard polymerase chain reaction (PCR) with HPV6, 11, 16 and 18 primers and found HPV11 and 16 DNA in two of 146 cases of OSCC (18). After both studies the authors concluded HPV as probably unimportant in the development of OSCC in those South African populations studied (17, 18).

The different detection methods and its various sensitivities seems to be the single most important reason for the wide variation in HPV prevalence reported in the literature. HPV cannot be cultured *in vitro* and detection of the virus relies on molecular analysis of HPV-DNA sequence using nucleic acid probes (13). Three types of nucleic acid hybridization methods for HPV detection exist. These include direct nucleic acid probe methods-like Southern blot and ISH, hybridization signal amplification which is an extension of direct probe techniques with sensitivity boost innovations-like multimeric layering of reporter molecules on DNA probes (GenPoint®), and target amplification methods, most notably PCR (13). Target amplification has been confirmed as the most sensitive of all DNA analysis techniques and real-time quantitative PCR as the most sensitive of the target amplification methods (19) with a one target nucleic acid sequence detection limit (13). Because of the proven differences in technique sensitivities, and the fact that a recent meta-analytic study found HPV16 to be a significant risk factor for OSCC (2), it was decided to compare the three types of nucleic acid hybridization methods for HPV detection in the current study to re-evaluate the prevalence and possible role of HPV in primary OSCC in a South African population sample.

Materials and methods

Sample collection

All resections of primary OSCC with neck lymph node dissections involving the lining mucosa anterior to the anterior tonsillar pillars, the masticatory mucosa of the gingiva and palate as well as the specialized mucosa of the tongue performed from 1998 to 2003 were retrieved from the archives of the Departments of Anatomical and Oral Pathology, University of Pretoria, South Africa.

Laboratory procedures

Tissue block selection

Haematoxylin and eosin (H & E)-stained sections of each case were retrieved and independently screened by two pathologists to confirm the histological diagnoses of OSCC. In each case a tissue block clearly showing the transitional zone between morphologically normal oral mucosa adjacent to infiltrating OSCC was chosen for the HPV studies.

In situ hybridization detection of HPV

About 5 μm sections of each tissue block was deparaffinized and hydrated sections were stained using Rembrandt (PanPath, Kruislaan 400, 1098 SM Amsterdam, the Netherlands) biotin-labelled pan-HPV probes and the Rembrandt Universal DISH and AP Detection Kit for biotin-labelled probes. Proteolysis was performed at 37°C for 30 min with proteolytic working solution. Sections were then dehydrated, air-dried, and coverslipped with 10–20 μl of biotin-labelled probe solution. Denaturation was performed at 95°C on a hotplate for 5 min. The sections were hybridized overnight at room temperature followed by washing with PanWash for HPV probes. Detection and staining was carried out according to the manufacturer's instructions. After the sections were incubated with antibiotin AP conjugate, staining with nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) was performed overnight at room temperature to visualize positive results. Sections were mounted with Dako® (Dako Corporation, Carpinteria, CA, USA) Faramount. Assessment of ISH signals was carried out with a light microscope. A tissue block from a confirmed HPV-positive cervix carcinoma was used as positive control. Dark brown to black punctate or diffuse nuclear staining was regarded as being positive for HPV.

ISH with signal enhancement (Genpoint®) for HPV detection

On all of the above sections CSAC-ISH was carried out using Dako GenPoint system (No. K0620). About 4 μm sections were deparaffinized and hydrated. Catalysed signal amplification colorimetric/*in situ* hybridization (CSAC-ISH) was performed as follows: heat target retrieval of sections was performed using a microwave, microwave pressure cooker, and citric acid buffer (pH 6). Once the pressure cooker had reached full pressure, the sections were treated for 3 min 20 s at medium high level. Digestion with 0.01% pepsin in 0.2 M HCl was carried out at room temperature for 13 min. Background quenching in all sections was performed by 0.3% hydrogen peroxide for 20 min. About 10–15 μl of wide spectrum HPV-biotinylated DNA probe reagent (DakoCytomation) was applied to each section and covered with coverslips. Target and probe DNA was denatured by incubating slides for 5 min at 95°C on a hotplate. Hybridization was performed in a humid chamber at 37°C overnight. After washing, the GenPoint Kit (No. K0620 Dako) was used according to the manufacturer's instructions. First sections were incubated with primary streptavidin-HRP in a dilution of 1:100 for 30 min and after washing, incubated with biotinyl tyramide solution for 15 min. This was followed by incubating with secondary streptavidin-HRP for 15 min and diaminobenzidine (DAB) chromogen for 7 min. Sections were counterstained with haematoxylin dehydrated and mounted with DPX mountant. A tissue block from a confirmed HPV-positive cervix carcinoma was used as positive control. Dark brown to black punctate or diffuse nuclear staining was regarded as being positive for HPV.

Polymerase chain reaction

HPV-DNA extraction of paraffin blocks. Three 10 μm sections were cut from each paraffin block and put into test tubes. After eight tissue blocks were cut, sections were taken from a negative control brain tissue block. The order in which the blocks and control sections were cut was documented and the extraction of the DNA was also performed in the same order. Sections were cut using a clean pair of gloves for each block. The microtome blade, chuck and knife holder were thoroughly cleaned with xylene and rinsed with alcohol between sectioning of the blocks. All forceps and utensils used were also cleaned in this manner. Sections from different blocks were each cut using a new unused area of the microtome blade to prevent contamination from the blade. Each microtome blade was therefore divided into halves. The sections were treated with xylene to remove the paraffin wax followed by absolute alcohol. The tissue pellets recovered were suspended in ATL buffer (Qiagen, Hilden, Germany) and digested with Proteinase K (Qiagen®) at

56°C for 3 h. DNA was extracted with a QIAamp® DNA Mini Kit (Qiagen) according to the manufacturer's instructions. DNA was extracted using DNAse-, RNAse- and pyrogen-free sterile filter tips. The necessary precautions were taken to prevent contamination throughout the procedure. DNA extraction of the tissue sections of the cases studied and all the brain tissue control blocks were carried out in the same order as in which they were sectioned. The extracted DNA was frozen at −20°C until PCR detection of HPV-DNA commenced.

Real-time PCR detection of HPV-DNA. Detection of HPV16 in the extracted DNA was performed using real-time quantitative PCR in an ABI Prism 7700 Sequence Detection System and the TaqMan Universal PCR Master Mix (PE Applied Biosystems, Perkin-Elmer, New York, NY, USA). PCR amplification was performed with 10 μl specimen in a total volume of 50 μl, using primers HPV E1 16L, HPV E1 16R/HPV E1 RE, and probe HPV16 (20). Tubes containing all PCR components but without template DNA, were included to ensure that the reagents were free of contamination. A plasmid-containing HPV16 (ATCC 45113D) was used as positive control and human brain was used as a negative control.

For HPV18 the method of Gravitt et al., (21) was adapted, using 900 nM of each primer HPV18-U and HPV18-L and 200 nM of the HPV18-TM probe in a total volume of 25 μl TaqMan Universal PCR Master mix with 2.5 μl of each specimen and amplification profile: 50°C, 2 min; 95°C, 10 min; 50 cycles of 95°C, 15 s; 60°C, 1 min. The HPV18 plasmid (ATCC 45152D) was used as positive control and human brain was used as a negative control.

Results

Fifty-nine cases of OSCC retrieved from the archives of the Anatomical and Oral Pathology laboratories of the University of Pretoria complied with the inclusion criteria of this study. The gender, age distribution, and race of the patients are listed in Table 1.

Only HPV18 DNA was detected in the primary tumours of seven patients (Table 1) using real-time PCR. No HPV signals were detected with ISH or amplified ISH techniques.

Table 1 The gender, age distribution, race, and metastatic disease profile of the patients as well as HPV results of the study

Race and gender	**Black male**	**Black female**	**White male**	**White female**	**Indian male**
Mean age (years)	54 (47–61)	45 (38–67)	62 (42–79)	60 (45–76)	47
Total patients	15	6	25	12	1
HPV16 (RT-PCR)	None	None	None	None	None
HPV18 (RT-PCR)	1	1	4	1	None
HPV16 (ISH)	None	None	None	None	None
HPV18 (ISH)	None	None	None	None	None
HPV, human papilloma virus; RT-PCR, real-time polymerase chain reaction; ISH, *in situ* hybridization.					

Discussion

The role of high-risk oncogenic HPV in pre-malignant and malignant oral lesions has been an issue of extreme controversy with conflicting data reported by numerous studies. Its possible role in the pathogenesis of OSCC has been extensively investigated and its prevalence in OSCC reported to vary from <5% to 100% (18, 22). A recently reported meta-analysis concluded HPV, specifically HPV16 to be a significant independent risk factor for OSCC (6). In our study, strong positive signals for the presence of HPV-DNA was found on all the positive controls as well as for HPV18 in seven primary tumours using real-time PCR (Table 1). It was however, not possible to determine whether the HPV-DNA was located in the tumour cells or in the adjacent mucosal epithelial cells, a well-known limitation of this technique when not used in conjunction with microdissection.

Although well-established as aetiological agents of anogenital carcinoma (2), the definite association of HPV with the mucosa of the oral cavity anterior to the anterior fauces and its pathological conditions remains uncertain. HPV has been shown to have a predilection for certain, especially non-keratinized anatomical sites in the oropharynx (7–9) and has, more importantly, been detected in clinically normal oropharyngeal mucosa which included high-risk viruses like HPV16 (4, 5, 23, 24). This has lead to other interesting opinions regarding its presence in OSCC lesions-like the oral cavity acting as a possible reservoir for HPVs (25) and the presence of HPV to be incidental colonization rather than true viral infection (26). The disappearance of HPV-DNA over time discovered in cervix carcinomas (27) indicate HPV to have possible early effects on cellular function that could lead to carcinogenesis (28) but this has not been investigated in OSCC. This may explain a low-HPV prevalence in early tumours but does not explain the high-HPV prevalence reported in some OSCC studies.

We confirmed real-time PCR as the most sensitive HPV detection method in our study as well as the findings of the previous two studies that reported a very low prevalence of HPV in OSCC in South African population samples. Neither the anatomical site of the primary tumour nor any of the patient variables-like race, gender or immune status seemed to influence the HPV status of the tumours in these patients. It is also possible that oral cancers in South Africa could be related to other types of HPV than types 16 and 18 and future PCR studies with HPV consensus primers to look for alternative types in these oral cancers might be worthwhile.

We therefore agree that HPV is probably unimportant in the development of OSCC (17) and hypothesize technical aspects of HPV detection as the main reason for a high incidence of positive results in many studies. In the recently reported meta-analysis that concluded HPV to be a significant independent risk factor for OSCC, only 38 of the 80 studies on HPV in OSCC performed some form of PCR for HPV detection but it was not possible to see how many studies used real-time PCR *per se* or ISH as it was grouped with immunoperoxidase and immunofluorescence studies for HPV detection (6). The technical difficulties encountered in interpretation of ISH results have previously been addressed by others (29). If diffuse positivity is encountered, it obviously poses no problems, but as HPV sometimes only have a 'punctate pattern' of positivity, described as 'discrete signal(s) in the nucleus' (30), there is always the possibility of it being non-specific staining only (29). None of the PCR-positive cases in our study was positive on either of the ISH techniques used. Even though we, like numerous other studies, confirmed real-time PCR as the most sensitive of the detection methods in our study, much consideration is needed when interpreting its results. PCR assays are by nature subject to environmental contamination and previously amplified material (amplicons) can contaminate negative specimens resulting in false-positive results (13). Stringent amplicon-containment procedures should therefore be implemented in all

laboratories and should include enzymatic amplicon elimination systems (13), regular negative control blocks such as human brain tissue incorporated after every five to eight tissue sections, clean gloves, and a new microtome blade for each tissue block. The chuck, knife holder, and all forceps and utensils used should be thoroughly cleaned with xylene and rinsed with alcohol between sectioning of the blocks. In this study, each microtome blade was divided in two halves and sections from different blocks were each cut using a new unused area of the microtome blade to prevent contamination from the blade. Using a new blade for each block is probably an even more certain way to exclude contamination. Whether HPV is only a regional commensal of the oral cavity or actually do become pathogenic in some cases resulting in carcinoma remains to be answered.

We conclude that future studies using real-time PCR with specific reference to the exact anatomical location and methods of contamination control might shed a whole different perspective on the actual relationship of HPV with the mucosa of the oral cavity.

References

1. Walker DM, Boey G, McDonald LA . The pathology of oral cancer. Pathology 2003; 35: 376– 83.

2. Clifford GM, Smith JS, Plummer M, Munoz N, Franceschi S . Human papillomavirus types in invasive cervical cancer worldwide: a meta-analysis. Br J Cancer 2003; 88: 63– 73.

3. Syrjanen S . Human papillomavirus infections and oral tumors. Med Microbiol Immunol (Berl) 2003; 192: 123– 8.

4. Terai M, Hashimoto K, Yoda K, Sata T . High prevalence of human papillomaviruses in the normal oral cavity of adults. Oral Microbiol Immunol 1999; 14: 201– 5.

5. Kurose K, Terai M, Soedarsono N, et al. Low prevalence of HPV infection and its natural history in normal oral mucosa among volunteers on Miyako Island, Japan. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2004; 98: 91– 6.

6. Miller CS, Johnstone BM . Human papillomavirus as a risk factor for oral squamous cell carcinoma: a meta-analysis, 1982–1997. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2001; 91: 622– 35.

7. Brandsma JL, Abramson AL . Association of papillomavirus with cancers of the head and neck. Arch Otolaryngol Head Neck Surg 1989; 115: 621– 5.

8. Syrjanen S . HPV infections and tonsillar carcinoma. J Clin Pathol 2004; 57: 449– 55.

9. Wilczynski SP, Lin BT, Xie Y, Paz IB . Detection of human papillomavirus DNA and oncoprotein overexpression are associated with distinct morphological patterns of tonsillar squamous cell carcinoma. Am J Pathol 1998; 152: 145– 56.

10. Gillison ML . Human papillomavirus-associated head and neck cancer is a distinct epidemiologic, clinical, and molecular entity. Semin Oncol 2004; 31: 744– 54.

11. Cruz IB, Snijders PJ, Steenbergen RD, et al. Age-dependence of human papillomavirus DNA presence in oral squamous cell carcinomas. Eur J Cancer B Oral Oncol 1996; 32B: 55– 62.

12. Kreimer AR, Alberg AJ, Daniel R, et al. Oral human papillomavirus infection in adults is associated with sexual behavior and HIV serostatus. J Infect Dis 2004; 189: 686– 98.

13. Hubbard RA . Human papillomavirus testing methods. Arch Pathol Lab Med 2003; 127: 940– 5.

14. Gillison ML, Koch WM, Capone RB, et al. Evidence for a causal association between human papillomavirus and a subset of head and neck cancers [see comments]. J Natl Cancer Inst 2000; 92: 709–20.

15. Mqoqi N, Kellett P, Madhoo J, Sitas F . Incidence of histologically diagnosed cancer in South Africa 1996–1997. In: National Cancer Registry of South Africa, 1996–1997. *Johannesburg, South Africa*: The National Cancer Registry, 2003; 52– 100.

16. Sitas F, Madhoo J, Wessie J . Incidence of histologically diagnosed cancer in South Africa, 1993–1995. In: National Cancer Registry of South Africa, 1993–1995. *Johannesburg, South Africa*: South African Institute for Medical Research, 1998; 13– 58.

17. Van Rensburg EJ, van Heerden WF, Venter EH, Raubenheimer EJ . Detection of human papillomavirus DNA with in situ hybridisation in oral squamous carcinoma in a rural black population. S Afr Med J 1995; 85: 894– 6.

18. Van Rensburg EJ, Engelbrecht S, Van Heerden WF, Raubennheimer EJ, Schoub BD . Human papillomavirus DNA in oral squamous cell carcinomas from an African population sample. Anticancer Res 1996; 16: 969– 73.

19. Gibson UE, Heid CA, Williams PM . A novel method for real time quantitative RT-PCR. Genome Res 1996; 6: 995– 1001.

20. Josefsson AM, Magnusson PK, Ylitalo N, Sorensen P, Qwarforth-Tubbin PK, Melbye M, et al . Viral load of human papilloma virus 16 as a determinant for development of cervical carcinoma *in situ*: a nested case-control study. Lancet 2000; 355: 2189– 93.

21. Gravitt PE, Peyton C, Wheeler C, Apple R, Higuchi R, Shah KV . Reproductibility of HPV 16 and HPV 18 viral load quantation using TaqMan real-time PCR assays. J Virol Methods 2003; 112: 23– 33.

22. Paz IB, Cook N, Odom-Maryon T, Xie Y, Wilczynski SP . Human papillomavirus (HPV) in head and neck cancer. An association of HPV 16 with squamous cell carcinoma of Waldeyer's tonsillar ring. Cancer 1997; 79: 595– 604.

23. Almadori G, Cadoni G, Cattani P, et al. Detection of human papillomavirus DNA in laryngeal squamous cell carcinoma by polymerase chain reaction. Eur J Cancer 1996; 32A: 783– 8.

24. Cox M, Maitland N, Scully C . Human herpes simplex-1 and papillomavirus type 16 homologous DNA sequences in normal, potentially malignant and malignant oral mucosa. Eur J Cancer B Oral Oncol 1993; 29B: 215– 9.

25. Remmerbach TW, Brinckmann UG, Hemprich A, Chekol M, Kuhndel K, Liebert UG . PCR detection of human papillomavirus of the mucosa: comparison between MY09/11 and GP5+/6+ primer sets. J Clin Virol 2004; 30: 302– 8.

26. Kansky AA, Poljak M, Seme K, et al. Human papillomavirus DNA in oral squamous cell carcinomas and normal oral mucosa. Acta Virol 2003; 47: 11– 6.

27. Evander M, Edlund K, Gustafsson A, et al. Human papillomavirus infection is transient in young women: a population-based cohort study. J Infect Dis 1995; 171: 1026– 30.

28. Ha PK, Califano JA . The role of human papillomavirus in oral carcinogenesis. Crit Rev Oral Biol Med 2004; 15: 188– 96.

29. Evans MF, Aliesky HA, Cooper K . Optimization of biotinyl-tyramide-based in situ hybridization for sensitive background-free applications on formalin-fixed, paraffin-embedded tissue specimens. BMC Clin Pathol 2003; 3: 2.

30. Cooper K, Herrington CS, Stickland JE, Evans MF, McGee JO . Episomal and integrated human papillomavirus in cervical neoplasia shown by non-isotopic in situ hybridisation. J Clin Pathol 1991; 44: 990– 6.