Assessing the nature of the association of human papillomavirus in oral cancer with and without known risk factors

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Background: Published studies assessing the association of human papillomavirus (HPV) and oral cancer, often include cases with additional confounders such as alcohol, tobacco, thus, precluding the assessment of HPV as a independent risk factor. Further apart from eliciting the mere presence of HPV, it is vital that the nature of the association is delineated for eliciting a causal inference. Thus, the present study assessed the presence and nature of the association of HPV in oral cancer cases with and without known risk factors.

Methods: The study compares the prevalence of HPV in oral cancer cases with known risk factors (group 1, n=15) to oral cancer (group 2, n=15) and oral epithelial dysplasia (group 3, n=15) cases with no known risk factors using PCR. Cases which are positive for HPV were subjected to p16 and p53 immunostaining to determine potential causal inference.

Results: HPV 16 was detected in only 1 case of group I, 3 cases of group II, and was absent in group III. HPV 18 was negative in all the 3 groups. All the HPV positive cases were negative for p16 and positive for p53 immunostaining.

Conclusions: Only a minor proportion of oral cancer cases without risk factors were positive for HPV 16. Even among these HPV 16 positive cases, the immunostaining profile (p53 positive and p16 negative) excludes the involvement of E6 and E7 mediated carcinogenesis. Thus even the weak HPV association noted in the present study cases may not be of causal nature.

Keywords: Human papillomavirus (HPV); oral squamous cell carcinoma (OSCC); oral epithelial dysplasia; risk factors; tobacco

Introduction

Most oral squamous cell carcinoma (OSCC) cases have known associated risk factors including tobacco, alcohol, areca nut, etc. In addition to this, there has been an ongoing debate as to the etiology of oral cancer in patients without any known risk factors. In such cases, a microbial etiology has been hypothesized. Human papillomavirus (HPV) a proven risk factor for cervical and oropharyngeal cancer

Table 1 Histopathological diagnosis wise distribution of cases in the study group

Histopathological	Group 1 (%)	Group 2 (%)	Group 3 (%)	Total (%)
Well differentiated OSCC	5 (33.33)	7 (46.67)	0	12 (26.67)
Moderately differentiated OSCC	9 (60.00)	6 (40.00)	0	15 (33.33)
Poor differentiated OSCC	1 (6.67)	2 (13.33)	0	3 (6.67)
Mild dysplastic lesion	0	0	5 (33.33)	5 (11.11)
Moderate dysplastic lesion	0	0	10 (66.66)	10 (22.22)
Total	15 (100.00)	15 (100.00)	15 (100.00)	45 (100.00)

OSCC, oral squamous cell carcinoma.

(1,2) has been closely associated with oral cancer, although conclusive evidence for causal inference is not established. Significant epidemiological evidence exists associating HPV and oral cancer, especially the high-risk types 16 and 18. Syrjänen *et al.*, in 1983 were the first to associate HPV with squamous cell lesions in various sites of the body including the oral cavity (3). Evidence for the possible association between HPV infection and oral cancer is provided by both in-vitro and in-vivo studies (4). Given that HPV is proven to be a causal factor in oropharyngeal cancer and based on epidemiological association with oral cancer, it is possible that HPV could be a potential risk factor for oral cancer.

Apart from HPV being a potential independent risk factor for oral cancer, it is also possible for a combined synergistic effect wherein the HPV infection predisposes the oral mucosa to the known carcinogen or vice versa. Thus, it is possible that patients with a history of tobacco usage and HPV have the additional risk of oral cancer (5,6). The present study aims to determine the association between HPV and OSCC with no known risk factors. HPV subtypes 16 and 18 were selected for the study due to their close epidemiological association with oral cancer (7). The mere presence of HPV in oral cancer does not constitute a causal role. Thus, in addition to establishing the presence of the virus, it is vital to assess the factors involved in the HPV mediated carcinogenesis. One such factor is p16. In HPV mediated carcinogenesis, HPV E-7 oncoprotein is shown to destabilize pRb which is responsible for suppressing p16 expression. Thus, tumor cells bypass the pRb-dependent cell cycle arrest resulting in marked overexpression of p16 (8,9). Similarly, in HPV mediated carcinogenesis, HPV E6 oncoprotein was found to induce FoxM1B by suppressing p53 resulting in loss of cell cycle regulation (10). Thus, in addition to detecting HPV through PCR, a positive p16 immunostaining and a negative p53 immunostaining would provide additional evidence for an HPV mediated carcinogenesis.

Methods

Ethical approval was obtained from the Dr. D. Y. Patil Vidayepeeth institutional ethics committee (Ref no. DYPDCH/EC-5/OP-35).

Informed consent was not applicable to the present study as the included specimens were from archival collection. Forty-five archival paraffin-embedded blocks of tissue specimens consisting of 30 OSCC cases and 15 oral epithelial dysplasia cases were collected.

The study samples were divided into the following groups:

❖ Group 1: 15 histopathologically diagnosed cases of OSCC with a known history of tobacco smoking;
❖ Group 2: 15 histopathologically diagnosed cases of OSCC with no known risk factors;
❖ Group 3: 15 histopathologically diagnosed cases of oral epithelial dysplasia (OED) with no known risk factors.

Group 1 consisted of 6 females and 9 males, group 2 comprised 3 females and 12 males group 3 comprised of 4 females and 11 males. The age range of the patients was 30–70 years. *Table 1* summarizes the distribution of cases based on the histopathological diagnosis.

DNA extraction from paraffin-embedded tissue

Sample block was trimmed to remove excess paraffin. Tissue sections were prepared to a thickness of 5–10 μm and were immediately transferred to 1.5 or 2 mL microcentrifuge tube to which 1 mL xylene was added. At room temperature, the sample was centrifuged for 2 minutes. Supernatant resulting from the centrifugation was pipetted leaving the

pellet behind. To the pellet, 1 mL ethanol (96–100%) was added and were mixed at room temperature using vortexing and centrifugation for 2 min. At 37 ℃, the samples were incubated for 10 minutes. 180 µL Buffer ATL was used to resuspend the pellet, following which 20 µL proteinase K was added and were vortexed and incubated for 1 hour at 56 ℃. Carefully without wetting the rim, the entire lysate was transferred to the QIAamp MinElute column (in a 2 mL collection tube). After closing the lid, centrifugation was carried out for 1 minute at 6,000 ×g (8,000 rpm). After opening the QIAamp MinElute column, 500 µL Buffer AW1 was carefully added without wetting the rim. After closing the lid, centrifugation was carried out for 1 minute at 6,000 ×g (8,000 rpm). QIAamp MinElute column was placed in a clean 2 mL collection tube, following which the collection tube containing the flow-through was discarded. Without wetting the rim, the QIAamp MinElute column was carefully opened and 500 µL Buffer AW2 was added, following which the lid was closed and centrifugation was carried out for 1 minute at 6,000 ×g (8,000 rpm). QIAamp MinElute column was placed in a clean 2 mL collection tube, following which the collection tube containing the flow-through was discarded. To dry the membrane completely, centrifugation was carried out at 20,000 ×g; 14,000 rpm for 3 min. A clean 1.5 mL microcentrifuge tube was used to place the QIAamp MinElute column, following which the collection tube containing the flow-through was discarded. After opening the lid of the QIAamp MinElute column, 20–100 µL Buffer ATE was applied to the center of the membrane. After closing the lid, it was incubated for 1 minute at room temperature (15–25 ℃). Centrifugation was carried out for 1 minute at 20,000 ×g; 14,000 rpm.

Polymerase chain reaction (PCR)

To determine the absence of PCR inhibitors and the integrity of the DNA, a 165 bp segment of a human β2-microglobin gene was amplified. HPV16/18 DNA was analyzed only for those samples that were positive for the β2-microglobin sequence. Primer pairs located in the E6 gene open reading frame (ORF) which are specific for HPV subtype were used. 1.0 µg of DNA in a total volume of 25 µL (1× Tris buffer, 1.5 mM $MgCl_2$, 1 µM of each primer, 200 µM each of dATP, dCTP, dGTP, dTTP and 1 U Taq) polymerase was used to perform the amplification reactions. In the DNA thermocycler (Eppendorf) 35 amplification cycles were performed with the following profile:

- ❖ Denaturation 1 minute at 95 ℃;

- ❖ Annealing 1 minute at 54 ℃;
- ❖ Elongation 2 minute at 72 ℃.

The last cycle was followed by a final extension of 72 ℃ for 7 minute. A negative control (Sterile water instead of DNA) and positive control (HPV16 positive Cervical and HPV 18 positive uterine carcinoma) was used during each PCR experiments. 1.5% agarose gel electrophoresis and ethidium bromide staining were used to analyze the PCR products. SPSS 17.0 version was used to analyze the resulting data.

Immunohistochemistry (IHC)

The cases which were positive for HPV in the PCR, were subjected to immunostaining for p16 and p53. IHC was performed on formalin-fixed paraffin-embedded sections of 3 µm thickness. The sections were deparaffinized and rehydrated by giving them 3 changes of xylene and various grade of alcohol. For antigen retrieval sections were boiled in 0.01 citrate buffer (pH, 6.0) for 10 minutes. The sections were incubated with p16[INK4a] primary mouse monoclonal antibody (Biogenex Lot no. AM5400315) and anti-p53(Clone D0-7, 1:200 dilution, DAKO A/S, CA, USA). The sections were then washed with Tris buffer and sliders were further incubated with poly horseradish peroxidase for 30 minutes. The slides were then reacted with diaminobenzene hydrochloride for 5 minutes and finally counterstained with Mayer's hematoxylin. For, p16 expression results were considered positive only with 70–75% nuclear and cytoplasmic staining (9). For p53 quantification was done as (+) mild, (++) moderate and (+++) intense expression of nuclear stain. *Figure 1* summarizes the workflow of the study.

Results

PCR

In group 1, only 1 case (6.67%) was positive for HPV 16, whereas, in group 2, 3 cases (20%) were positive for HPV 16. There was no statistically significant difference in the prevalence of HPV 16 between group 1 and 2 (*Table 2*). There were no HPV positive cases in group 3. HPV 18 was negative in all the 3 groups. *Table 3* provides data on site-specificity of HPV in OSCC. *Table 4* provides data on OSCC grade based specificity of HPV in OSCC.

IHC

All the HPV positive cases subjected to immunostaining
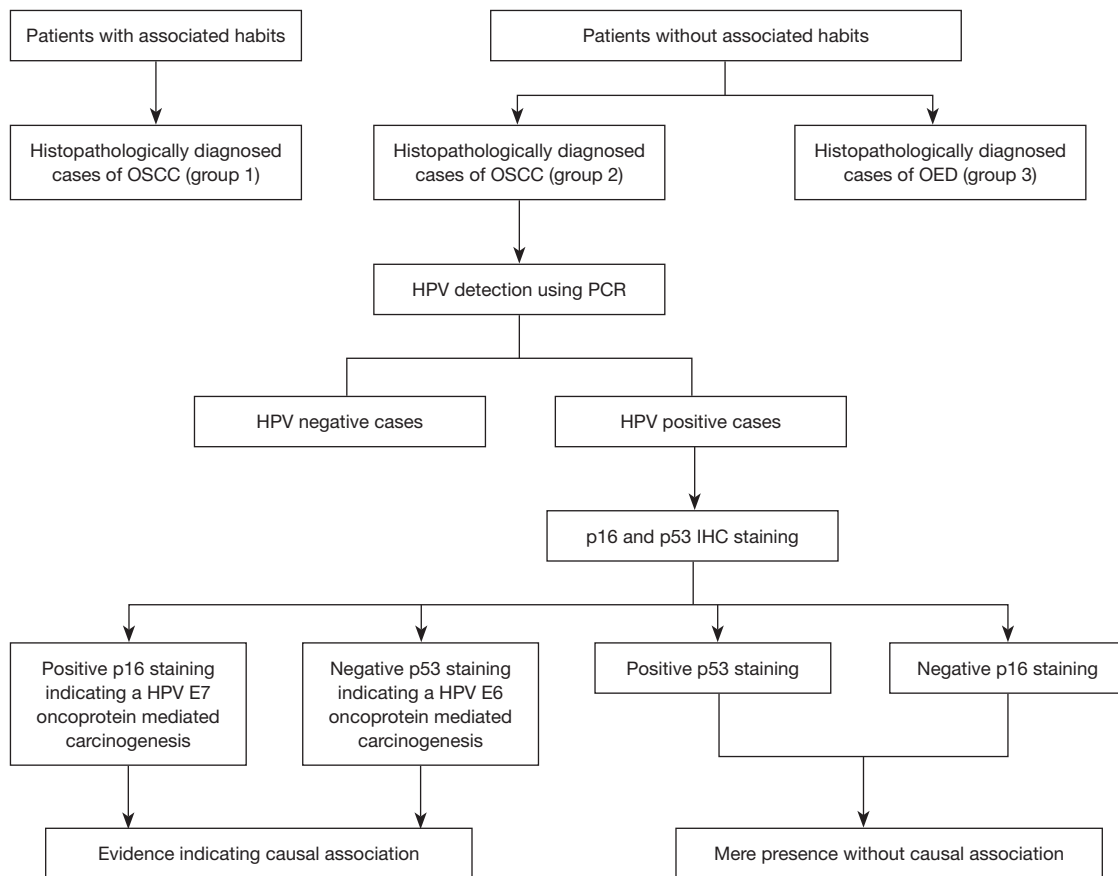
Figure 1 Study workflow. OSCC, oral squamous cell carcinoma; HPV, human papillomavirus.

Table 2 Comparison of the presence of HPV among the group 1, 2 and 3

Group	n	HPV
Group 1	15	1 (6.67)
Group 2	15	3 (20.0)*
Group 3	15	0[#&]

*: compare with group 1, Z=1.10, P>0.05; #: compared with group 1, Z=1.04, P>0.05; &: compared with group 1, Z=1.94, P>0.05. HPV, human papilloma virus.

**Table 3** Site specificity of HPV in OSCC

Site	Presence of HPV		
	Group 1 (n=15)	Group 2 (n=15)	Group 3 (n=15)
Buccal mucosa	1 (6.67%)	0	0
Gingiva	0	2 (13.33%)	0
Tongue	0	1 (6.67%)	0

HPV, human papillomavirus; OSCC, oral squamous cell carcinoma.

Table 4 OSCC grade specificity of HPV in OSCC

Histopathological grade	Presence of HPV		
	Group 1 (n=15)	Group 2 (n=15)	Group 3 (n=15)
Well differentiated OSCC	0	2 (13.33)	0
Moderately differentiated OSCC	1 (6.67)	1 (6.67)	0

OSCC, oral squamous cell carcinoma; HPV, human papillomavirus.
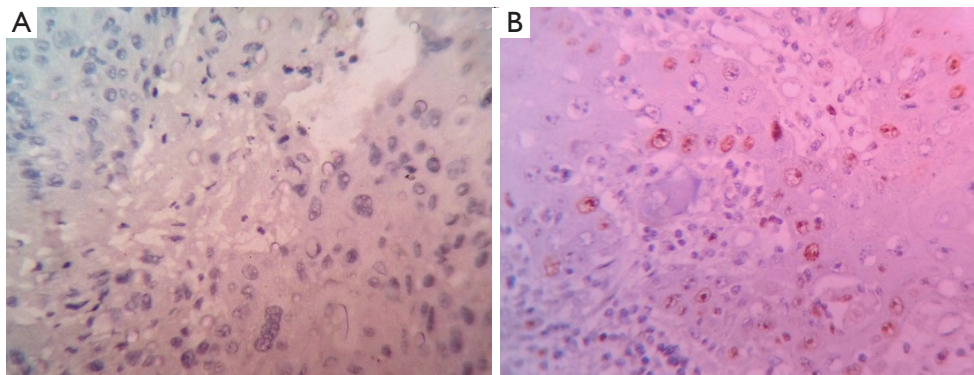
Figure 2 p16 and p53 immunostaining pattern in HPV positive cases. (A) Absence of p16 immunostaining (×40); (B) intense nuclear p53 immunostaining (×40). HPV, human papillomavirus.

were negative for p16 and positive for p53 (*Figure 2A,B*).

Discussion

The OSCC incidence and mortality rate has shown a steady increase, with 6.6/100,000 and 3.1/100,000 being the age-standardized incidence and mortality in men and 2.9/100,000 and 1.4/100,000 being the age-standardized incidence and mortality in women (11). The increasing prevalence of oral cancer is presumed to be the result of the increasing use of known risk factors such as alcohol, tobacco, areca nut, etc. (7). Despite most of the oral cancer cases being associated with these well-established risk factors, there are reported cases wherein the associated risk factor is not identified. Recent studies have explored the role of potential risk factors such as diet, immunity, infection in oral cancer, with special emphasis given to HPV 16 and 18 (12,13). Syrjänen *et al.* observation of the cytopathic effects of HPV (koilocytosis) in oral lesions under light microscopy was the first substantial evidence for a potential link between OSCC and HPV (3). The presence of HPV DNA in oral potentially malignant lesions (4/5 leukoplakias) and malignancies (3/6 carcinomas) was later confirmed using *in situ* hybridization, based on which Loning et al suggested a potential causal association between HPV and oral cancer (14). Syrjänen *et al.* (3) findings were substantiated by a number of studies including that of Wilczynski *et al.* who reported altered cytologic features in HPV infection with an additional feature of lack of keratin (15). Münger *et al.* reported that oral epithelial cells can attain immortality if they are infected with HPV 16 infection and are expressing E6 and E7 genes (16).

The above-mentioned studies support the hypothesis that HPV may not be specific to the anogenital epithelium and could be closely associated with oral epithelium as well (17). The prevalence of HPV in normal oral mucosa, oral potentially malignant disorders and oral malignancies varies widely from 0 to 70%, 0 to 85%, and 0 to 100%, respectively (18). The variations could, in turn, be the result of using diagnostic modalities with varying sensitivity and specificity. Based on the data from the above-mentioned studies, we hypothesized that although HPV could be present in both oral cancer cases with and without risk factors, a causal association is more likely in oral cancer without risk factors.

The HPV prevalence in all three groups of the present study sample was low, which is in contrary to several studies which have isolated HPV in OSCC at a relatively larger proportion. Ha *et al.* detected HPV in 86.2% of hyperplasia, 100% of dysplasia and 94.7% OSCC cases (19). Bouda *et al.* found HPV-16 DNA in 65% of dysplasia and 35% OSCC cases (20). Sugiyama *et al.* detected HPV in 74% of OSCC samples (21,22). The absence of HPV 16 and 18 in all the cases of OED in the present study is in contrast to several other studies including that of McCord *et al.* and Mravak-Stipetić *et al.* (23,24). Both these found a close association between HPV and OED with McCord *et al.* (23) observing diffuse loss of squamous differentiation and a high proliferation index in cases of severe epithelial dysplasia/carcinoma *in situ* indicating a close association to HPV and Mravak-Stipetić *et al.* (24) detecting HPV in 17.7% of oral dysplastic lesions.

The nature of association of the HPV with oral cancer was assessed by subjecting HPV positive cases to p53 and p16 immunostaining. In an HPV mediated carcinogenesis, the destabilization of the pRB by the HPV E-7 oncoprotein results in increased expression of p16 (8,9). In the present

study, all the cases positive for HPV in the PCR were negative for the p16, thus excluding an E-7 oncoprotein mediated carcinogenesis. An alternated pathway of HPV induced carcinogenesis is through E-6 oncoprotein mediated suppression of p53. In the present study, all the HPV positive cases were positive for p53. Thus, even among the minor proportion of HPV 16 positive cases, the immunostaining (p53 positive and p16 negative) infers that the association may not be of causal nature.

Conclusions

Among cases with no known risk factors only 3 OSCC cases were positive for HPV 16. None of the OED cases with no known risk factors were positive for HPV 16 or 18. Among the cases with known risk factors, only 1 OSCC case was positive for HPV 16. There was no statistically significant difference between the groups. Based on the results HPV 16 can only be weakly associated with oral cancer cases with and without known risk factors. The PCR results in combination with the absence of p16 and the presence of p53 immunostaining indicate that even the minimal HPV presence may not be of causal nature in the present study sample

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Footnote

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