

# Association of Human Papillomavirus Infection in Healthy Oral Mucosa, Oral Dysplasia, and Oral Squamous Cell Carcinoma

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## Abstract

**Aims:** Human papillomavirus (HPV) is an important risk factor for development of oral cancer; however, the integrational status of the virus into the host DNA association between HPV infection and oral squamous cell carcinoma (OSCC) is still uncertain has not been investigated to the same extent. The objective of the present study was to investigate the prevalence of consensus HPV, and HPV-16 and its integration status, in healthy oral mucosa, oral epithelial dysplasia (OED), and OSCC samples.

**Materials and Methods:** The study material consisted of 26 fresh, normal oral mucosa samples, and 53 and 27 paraffin-embedded OED and OSCC samples, respectively. The specimens were DNA extracted and investigated for the presence of HPV, and HPV-16 and its integration status, by polymerase chain reaction (PCR) and DNA sequencing.

**Results:** Thirty-eight (72%) of the 53 paraffin-embedded OED samples, 16 (59%) of the 27 OSCC samples, and 12 (46%) of the 26 control samples were found to be HPV-DNA positive, with nested PCR (NPCR). Further, HPV-16 was detected in 31 (82%), 15 (94%), and 0 (0%) HPV-positive OED cases, HPV-positive OSCC cases, and controls, respectively. Integration was observed in 26/31 (84%) and 13/15 (87%) of the HPV-16-positive OED and OSCC cases, respectively. A statistically significant difference was found comparing prevalence of HPV-16 in controls with that in both OED and OSCC samples ( $P < 0.005$ ). A statistically significant difference was also found comparing prevalence of integrated and episomal viral forms comparing OED and OSCC samples ( $P < 0.005$ ).

**Conclusion:** The high prevalence of HPV and high-risk HPV-16 in OED and OSCC samples suggests a potential aetiologic role for the virus in OSCC.

*Key Words: HPV, HPV-16, OSSC, OED, Episomal, Integration, PCR*

## Introduction

Squamous cell carcinoma (OSCC) is a major health problem. Prevalence is highest in developing countries, and men are affected to a greater extent than women [1]. Smoking, smokeless tobacco and alcohol consumption are considered important aetiological factors [2]. In addition to this, certain viral infections play an important role in malignancy [3,4]. Increasing evidence has suggested that infection with human papillomavirus (HPV) causes several cancer types. High-risk HPV genotypes (16, 18, 31, 33, 35) have been reported to be associated with OED and OSCC [5-10]. The relationship between HPV and the oral mucosa has been supported by several investigators reporting the presence of HPV DNA in healthy oral mucosa [11,12] as well as in OSCC [13]. Several meta-analyses indicate that HPV is detected with increased frequency in oral dysplastic and carcinomatous epithelium in comparison with normal oral mucosa [13].

In benign lesions the virus occurs in its circular form, called episomal – not integrated into the host cell genome, and in a large number of copies. In malignant lesions, it is integrated into the host cell genome. Notwithstanding this, it is possible to find episomal forms in the malignant cells and, once integrated, the virus cannot be reverted to its episomal forms [14]. Integration of HPV-DNA disrupts or deletes the E2 region, which results in loss of its expression, leading

to enhanced expression of viral oncogenes E6 and E7 [15]. High-risk HPV infection contributes to carcinogenesis and tumour progression through the two viral oncogenes E6 and E7 [16]. These oncogenes inhibit the activities of the p53 and retinoblastoma (Rb) protein and have been considered as an important feature in disrupting cell-cycle regulatory pathways, leading to a genetic progression to OSCC [17].

## Aims

The aim of the present study was to evaluate the prevalence of consensus HPV, and HPV-16 and its integration status, in healthy oral mucosa, OED, and OSCC samples, and to determine whether HPV in the oral cavity may play a role in the onset of oral dysplasia and in the transition towards squamous cell carcinoma.

## Materials and Methods

The study was carried out on 53 OED and 27 OSCC paraffin-embedded cases and 26 fresh tissue samples from clinically healthy Swedish volunteers. The patients' age range for OED was from 23 to 93 years (34 male, 19 female, mean age=65 years, SD=15), for OSCC from 20 to 84 years (20 male, 7 female, mean age=63 years, SD=16), and for normal oral mucosa (14 male, 12 female, mean ages 62 years, SD=15).

## Tissue specimens

### Paraffin-embedded tissue

Local anaesthesia was used to obtain biopsy specimens, which were taken from the lesion. Specimens were placed in 99% alcohol and kept at room temperature for 24 hours before being stored at  $-20^{\circ}\text{C}$  until analysed. For confirmation of the clinical diagnosis, histopathological examination was performed. Specimens were obtained from the Department of Oral and Maxillofacial Surgery at Gothenburg University.

### Fresh tissue

Local anaesthesia (lidocaine 20 mg/mL+12.5  $\mu\text{g}$  adrenaline; Astrazeneca, Södertälje, Sweden) was used to obtain biopsy specimens, which were taken from normal oral mucosa during dentoalveolar surgery. The biopsy specimens were rinsed twice in buffered saline. The specimens were placed in 99% alcohol and kept at room temperature for 24 hours before being stored at  $-20^{\circ}\text{C}$  until analysed. Specimens were obtained from the Department of Oral and Maxillofacial Surgery at Uppsala University. Informed consent was obtained from all volunteers. The volunteers had no ongoing history of HPV-associated diseases. The study was approved by the Ethics Committee of Uppsala University.

### DNA extraction

#### Paraffin-embedded tissue

All of the tissue specimens were fixed with formalin and embedded in paraffin. Ten 5  $\mu\text{m}$  sections were cut from each paraffin block. As previously described by Jalouli et al. [18], paraffin was dissolved with xylene, and digestion of tissues was done with proteinase K. DNA was purified by sequential phenol/chloroform extraction and salt/ethanol precipitation. DNA was dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). DNA concentrations and DNA quality were measured using Nanodrop. All DNA samples were tested by PCR with a housekeeping gene and were positive for  $\beta$ -actin.

#### Fresh tissue

Total DNA was extracted from fresh oral biopsies using the QIAamp tissue DNA Mini Kit manufacturer's protocol

**Table 1.** The primer sequences used in the PCR reactions.

Virus	size(bp)	Primer sequence
MY 11	450	5' GCM CAG GGW CAT AAY AAT GG 3'
MY 9		5' CGT CC MARR GGA WAC TGA TC 3'
		M=A+C, R=A+G, W=A+T, Y=C+T
GP5+	150	5' TTTGTTACTGTGGTAGATACTAC3'
GP6+		5' GAAAAATAAACTGTAAATCATATTC3'
HPV16 F	152	5' ATTAGTGAGTATAGACATTA3'
HPV 16 R		5' GGCTTTTGACAGTTAATACA3'
E2 F	351	5' CTTGGGCACCGAAGAAACAC3'
E2 R		5' TTGGTCACGTTGCCATTAC3'
$\beta$ -actin 1	254	5' GTG CTT TGA TGG AAG TTG AGG TAG 3'
$\beta$ -actin 2		5'GAG CGG GAAATC GTG CGT GAC ATT 3'

(QIAgen, Hilden, Germany). Briefly, tissue samples were weighed, cut into small pieces, and incubated at  $56^{\circ}\text{C}$  by addition of 180  $\mu\text{L}$  of ATL buffer supplied with 20  $\mu\text{L}$  of proteinase K per 25 mg of sample. When tissues were completely lysed, a volume of 200  $\mu\text{L}$  of lysate was transferred into a 2 mL microcentrifuge tube, and DNA extraction with QIAamp Mini spin columns was carried out using a QIAcube automate. Final elution of DNA extracted from tissue samples was performed with 200  $\mu\text{L}$  of double-distilled water. DNA concentrations and DNA quality were measured using Nanodrop. All DNA samples were tested by PCR with a housekeeping gene and were positive for  $\beta$ -actin.

### HPV single PCR

A single PCR assay was used to detect HPV. The samples were screened for the presence of HPV using the standard single PCR approach consisting of the MY09/MY11 primer set described by Jalouli et al. [18], (Table 1). Each PCR mixture was diluted with 2.5  $\mu\text{L}$  ten times PCR buffer (500 Mm KCl, 100 Mm TRIS-PH 8.5), 0.6  $\mu\text{L}$  ten times mix dNTP (25 $\mu\text{M}$ ), 3.5  $\mu\text{L}$   $\text{MgCl}_2$  (25 mM), 0.3  $\mu\text{L}$  oligonucleotide MY09 primer (100 mM), 0.3  $\mu\text{L}$  oligonucleotide MY11 primer (100 mM), and 14.2  $\mu\text{L}$   $\text{H}_2\text{O}$  to a final volume of 21.4  $\mu\text{L}$ . In addition, 3.5  $\mu\text{L}$  of the sample and 0.15  $\mu\text{L}$  of the Taq polymerase (0.75 U, AmpliTaq DNA polymerase, Applied Biosystems, Foster City, CA, USA) were added to the reaction mixture. Each cycle consisted of 1 min at  $95^{\circ}\text{C}$ , 1 min at  $55^{\circ}\text{C}$ , and 2 min at  $72^{\circ}\text{C}$ . A final extension step at  $72^{\circ}\text{C}$  was carried out for 5 min, and then at  $4^{\circ}\text{C}$ . Five microlitres of the amplified DNA was used as the template for the second PCR with the GP5+/GP6+ primer pair. The primer sequences used in the PCR reactions are shown in Table 1.

### HPV nested PCR

For HPV DNA detection by nested PCR (NPCR), two pairs of primers, GP5+/GP6+, were used. The DNA amplifications were performed in  $5 \times 1$  of PCR buffer, 2 mM  $\text{MgCl}_2$ , 0.2 mM dNTP, 2 pmol of primer GP5+/GP6+, and 1 U of AmpliTaq. The thermocycler temperature programme consisted of denaturation at  $95^{\circ}\text{C}$  for 30 s, annealing at  $45^{\circ}\text{C}$  for 30 s, and extension at  $72^{\circ}\text{C}$  for 1 min for 35 cycles. Each PCR was initiated by a 5 min denaturation step at  $95^{\circ}\text{C}$  and finished by a 10 min extension step at  $72^{\circ}\text{C}$ . The PCR assays were performed using Gene Amp PCR System 9700 (PE Applied Biosystems, Foster City, CA, USA), and the PCR products were analysed on 2% agarose gels. Two controls were used for each PCR assay reaction, described as negative control (containing all reagents except the template DNA) and positive control (DNA HeLa cells).

### Single PCR assay for HPV-16 DNA

A single PCR assay was used to detect HPV-16. The primer set used for detection of HPV-16 is described in Table 1. PCR reaction was carried out in 25  $\mu\text{L}$  reaction volume, and the conditions were as follows: step of denaturation at  $95^{\circ}\text{C}$  for 30 sec, annealing at  $52^{\circ}\text{C}$  for 45 sec, and elongation at  $72^{\circ}\text{C}$  for 45 sec for 35 cycles, with an initial incubation at  $95^{\circ}\text{C}$  for 10 min and a step of final elongation at  $72^{\circ}\text{C}$  for 10 min. Two controls were used for each PCR assay reaction, described as negative control (containing all reagents except the template DNA) and DNA from SiHa cells as positive control.

### Single PCR assay for integrated HPV-DNA

The integration of the HPV into the host DNA was detected

by PCR with E2-specific primers [19] (Table 1). PCR reaction was carried out in 25  $\mu$ L reaction volume, and the conditions were as follows: step of denaturation at 94°C for 1 minute, annealing at 57°C for 1 minute, and elongation at 72°C for 1 minute for 40 cycles, with an initial incubation at 94°C for 5 min and a step of final elongation at 72°C for 7 min [19]. Two controls were used for each PCR assay reaction, described as negative control (containing all reagents except the template DNA) and positive control (DNA from SiHa cells).

### Gel electrophoresis

Aliquots of 15  $\mu$ L of the PCR product were analysed on 2% agarose gel (DNA Agar; Marine Bio Products Inc., Quincy, MA, USA) containing 0.5  $\mu$ mol of ethidium bromide (Merck KGaA, Darmstadt, Germany), and visualized under ultraviolet light. The size of the amplified product was determined by comparison with a base-pair (bp) ladder size marker (Gene Ruler, 100 bp, 50 bp DNA Ladder Plus, Fermentas, St Leon-Rot, Germany).

**Table 2.** Age and gender of the control, OED and OSSC patients.

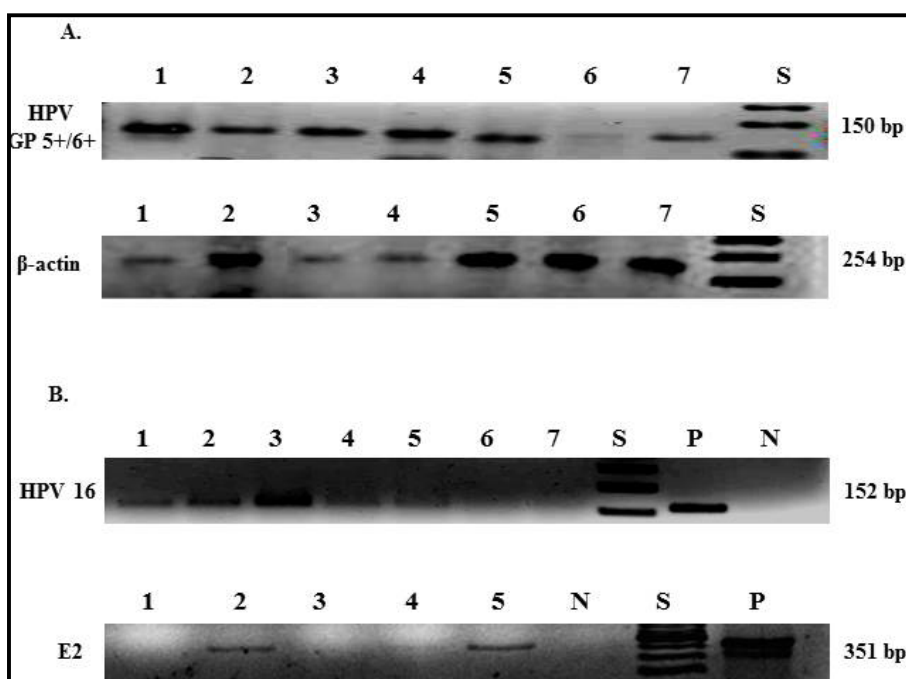
		<b>Kontroll</b> <b>(N=26)</b>	<b>OED</b> <b>(N=53)</b>	<b>OSSC</b> <b>(N=27)</b>	<b>Total</b> <b>(N=106)</b>	<b>P-value</b>
Age	Mean (SD)	61.54 (14.77)	64.85 (14.48)	63.07 (15.79)	63.58 (14.82)	0.638
	Median	63.5	69	64	66	
	Range	25 to 86	23 to 93	20 to 84	20 to 93	
	n	26	53	27	106	
Gender	n (%)					
<i>female</i>		12 (46.15)	19 (35.85)	7 (25.93)	38 (35.85)	0.346
<i>male</i>		14 (53.85)	34 (64.15)	20 (74.07)	68 (64.15)	

**Table 3.** Prevalence of HPV-DNA, HPV-16, and integrated viral forms in different location in OED, OSCC and control and grade of dysplasia in OED and histological grades of OSCC and control samples.

	Anatomic site				
	Floor of mouth	Tongue	Lip	Buccal	Gingiva
Graded dysplasia OED (n=53)					
<i>Mild dysplasia</i>	0	2	0	2	3
<i>Moderate dysplasia</i>	3	11	1	9	3
<i>Severe-dysplasya</i>	5	2	3	6	3
HPV-DNA + ; OED (n=38)					
<i>Mild dysplasia</i>	0	2	0	1	1
<i>Moderate dysplasia</i>	3	8	1	6	2
<i>Severe-dysplasya</i>	3	2	2	5	2
HPV 16 + ; OED (n=31)					
<i>Mild dysplasia</i>	0	1	0	1	1
<i>Moderate dysplasia</i>	1	8	1	5	2
<i>Severe-dysplasya</i>	3	2	1	3	2
Integration : OED (n=26)					
<i>Mild dysplasia</i>	0	1	0	1	1
<i>Moderate dysplasia</i>	0	7	0	5	2
<i>Severe-dysplasya</i>	3	1	1	2	2
Histological grades of OSCC (n=27)					
<i>Poor-differentiated</i>	3	1	0	0	0
<i>Moderately-differentiated</i>	4	3	0	3	2
<i>Well-differentiated</i>	2	4	0	4	1
HPV-DNA + ; OSCC (n=16)					
<i>Poor-differentiated</i>	2	1	0	0	0
<i>Moderately-differentiated</i>	1	2	0	2	1
<i>Well-differentiated</i>	2	2	0	2	1
HPV 16 + ; OSCC (n=15)					
<i>Poor-differentiated</i>	2	0	0	0	0
<i>Moderately-differentiated</i>	1	2	0	2	1
<i>Well-differentiated</i>	2	2	0	2	1
Integration; (n=13)					
<i>Poor-differentiated</i>	2	0	0	0	0
<i>Moderately-differentiated</i>	1	1	0	2	1
<i>Well-differentiated</i>	2	2	0	1	1
HPV-DNA + ; Control (n=26)	0	0	0	0	12
HPV-16 + ; Control (n=12)	0	0	0	0	0

**Table 4.** Prevalence of HPV-DNA, HPV-16, integrated and episomal in control, OED and OSCC samples.

		Control (N=26)	OED (N=53)	OSCC (N=27)	Total (N=106)	P-value
HPV	n (%)					
neg		14 (53.85)	15 (28.30)	11 (40.74)	40 (37.74)	0.083
pos		12 (46.15)	38 (71.70)	16 (59.26)	66 (62.26)	
HPV-16	n (%)					
neg		26 (100.00)	7 (18.42)	1 (6.25)	34 (42.50)	<0.001
pos		0 (0.00)	31 (81.58)	15 (93.75)	46 (57.50)	
Episomal	n (%)					
neg			26 (83.87)	13 (86.67)	39 (84.78)	1.000
pos			5 (16.13)	2 (13.33)	7 (15.22)	
Integration	n (%)					
neg			5 (16.13)	2 (13.33)	7 (15.22)	
pos			26 (83.87)	13 (86.67)	39 (84.78)	1.000



**Figure 1.** Representative results of PCR for HPV-DNA, HPV-16 and E2 integration. A. Amplicon obtained by PCR using primers specific for  $\beta$ -actin and HPV consensus primers (GP5+/6+). B. Amplification of the samples positive for consensus primers by HPV-16 and E2 gene in the samples positive for HPV-16. Lane p represents positive control and lane N represents the negative control. Examples of viral genomic integration (E2: lanes 1 and 3) and of viral episomal forms (E2: lanes 2, 4 and 5).

### Sequencing of the DNA PCR product

The products from the HPV-positive OSCC samples were sequenced. Direct DNA sequence analysis was performed using a capillary sequencer (ABI Prism 310, PE Applied Biosystems, Carlsbad, CA, USA), with MY and GP primer sets for HPV-DNA. Sequencing products were purified of unincorporated dye-labelled dideoxynucleotides by processing through Centri-Sep spin columns (PE Applied Biosystems, Carlsbad, CA, USA). Sequence analysis was automatically performed on the ABI Prism-310 Genetic Analyzer. We used the basic local alignment search tool (BLAST).

### Statistical analysis

Statistical analyses were performed using the SPSS software package (SPSS for Windows, version 16.0; SPSS, Inc., Chicago, IL, USA). P-values and 95% confidence intervals (CI) were calculated using Anova and Fisher's exact test. Results were considered significant if the p-value was less than 0.05 (5%).

## Results

### Study groups

A total of 26 healthy, fresh oral mucosa samples, and 53 and 27 paraffin-embedded OED and OSCC samples, respectively, were analysed for detection of HPV-DNA, and high-risk HPV-16 and its integration status, by PCR and NPCR methods. The cases and controls were statistically comparable with respect to gender and mean age. There was no significant difference between male and females with respect to staging and grading among the groups (Table 2).

### Prevalence of HPV

Twelve (46%) of the 26 healthy oral mucosa, 38 (72%) of the 53 paraffin-embedded OED samples, and 16 (59%) of the 27 OSCC samples were found to be HPV-DNA positive, using NPCR (Table 3) (Table 4, Figure 1A). No significantly difference of HPV-DNA prevalence was found between the groups. Further, the samples positive for HPV infection were tested for HPV-16, which was detected in 0/26 (0%), 31/38 (82%), and 15/16 (94%) of the healthy oral mucosa

and the OED and OSCC cases, respectively ( $P < 0.005$ ) (Table 4, Figure 1B). Prevalence of HPV-DNA and HPV-16 in different location and grade of dysplasia in OED and histological grades of OSCC and control samples are shown in the Table 3.

#### HPV integration

Integration of the HPV into the host DNA was detected by PCR with E2-specific primers. If the virus is integrated, the E2 oncogene will be disrupted; hence, the presence of HPV infection with loss of E2 was considered indicative of integration. Integration was observed in 27/31 (87%) and 13/15 (87%) of the HPV-16 positive OED and OSCC cases, respectively (Table 4, Figure 1B). A statistically significant difference was also found in comparing prevalence of integrated and episomal viral forms in both OED and OSCC samples ( $P < 0.005$ ) (Table 4). For the detection of integration the E2 open reading frame of HPV-16 was amplified (Figure 1B). Prevalence of integration in different location and grade of dysplasia in OED and histological grades of OSCC and control samples are shown in the table 3.

#### Sequencing

The products from the HPV-positive samples were sequenced with MY and GP primer sets for HPV-DNA. For DNA versus DNA comparison, the Basic Local Alignment Search Tool (BLAST; <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) program is typically used to find identical sequence regions in a database. We used this database to find the homology of the HPV positive PCR products, and found 100% homology.

### Discussion

In this study, the prevalence of human papillomavirus was examined in healthy, fresh oral mucosa and paraffin-embedded biopsies obtained from patients with the clinical diagnoses OED and OSCC, by highly sensitive PCR method. No significant difference in HPV-DNA prevalence was found between the groups. Presence of HPV in the oral mucosa has previously been reported in healthy oral mucosa [13] as well as in OSCC [12]. The presence of HPV in normal mucosa of the oral cavity has also been reported previously in several studies and the prevalence varies considerably between different regions; in Japan 81% [11] in Iran 6,1% [20], and in Pakistan 24,5% [21]. According to a literature review, the prevalence of HPV in normal oral mucosa ranged from 0% to 60% [22].

We observed a statistically significant difference in comparing the prevalence of integrated and episomal viral forms between the OED and OSCC samples. A statistically significant difference was also found when comparing the prevalence of HPV-16 in controls compared with OED and OSCC samples. The prevalence of HPV in precancerous and cancerous oral lesions in previous reports by other investigators varies widely from 0% to 100% [23-26]. High-risk HPV-16 has been clearly shown to be the dominant type

in head and neck cancers [27-29]. HPV has been found to be both in an episomal form and in an integral form. It has also been suggested that HPV may be latent for a long time in the episomal format in the oral mucosa, hence being responsible for initiation and development of tumoral growth. This tumoral growth may occur as a result of a multicarcinogenic interaction with some other carcinogens [30-32]. The E2 open reading frame (ORF) has been identified as the preferential site of viral integration because it has been found to be disrupted or deleted more frequently than other sites [33-37].

Integration is thought to induce a deregulation of the cell-cycle control and therefore uncontrolled cellular proliferation, dependent on constitutive expression of the viral oncogenes E6 [38]. In this study HPV-16 was detected in 82% and 94% of HPV-positive OED and OSCC samples, respectively. When these samples were analysed for integration, in 84% and 87% of HPV-16-positive OED and OSCC samples, respectively, the E2 gene PCR product could not be detected, suggesting that the virus was integrated into the cellular genome of these HPV-16-positive cases.

According to previous studies, integration of HPV-16 DNA correlates with a selective growth advantage and may allow cancer cells to grow out of its competitors; it can be an important step for oncogenesis [39]. Therefore, the measurement of HPV-16 integration would be a complementary tool for the assessment and identification of patients at risk of developing squamous cell carcinoma.

There are several methods with varying sensitivity and specificity that can be used to detect the prevalence of HPV, and HPV-16 and its integration status. In some studies high variation in integration frequency has been reported in head and neck squamous cell cancer HNSCC, ranging from virus being present only in an episomal form to its being 100% integrated, depending on the techniques applied to determine integration status [40,41].

In this study, qualitative PCR was used, a sensitive method that allows the detection of small amounts of DNA and can be confirmed with sequencing. In this study we have used nested PCR, the superior method for detecting HPV-DNA [42]. Many studies have shown that the nested PCR method is a sensitive and useful tool for HPV-DNA detection compared with single PCR [43,44].

In conclusion our data indicate a rise in the HPV and HPV-16 detection in OED and OSCC. This finding supports the involvement of HPV-16 and its integration to the host genome. The presence of HPV, in the cases of OED and OSCC, suggests that the virus may play an aetiological role in carcinogenesis in the oral cavity in the development of some oral carcinomas.

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