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

**Miranda M. Jalouli1, Jamshid Jalouli1, Bengt Hasséus2, Jenny Öhman2, Jan-M. Hirsch1, and Lars Sand1**

*1Department of Surgical Sciences, Oral and Maxillofacial Surgery, Uppsala University, Sweden*

*2Department of Oral Medicine and Pathology, Institute of Odontology, Sahlgrenska Academy at the University of Gothenburg, Gothenburg, Sweden*

Correspondence to: Lars Sand, Department of Surgical Sciences, Oral and Maxillofacial Surgery, Medical Faculty Uppsala University, SE-751 85 Uppsala, Sweden. Tel: +46186116450; Fax:+4618559129; e-mail: [Lars.Sand@surgsci.uu.se](mailto:Lars.Sand@surgsci.uu.se)

**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 OSSC 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 OSSC 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 OSSC cases, and controls, respectively. Integration was observed in 26/31 (84%) and 13/15 (87%) of the HPV-16-positive OED and OSSC 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 OSSC samples suggests a potential aetiologic role for the virus in OSCC.

### Keywords: 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) 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 OSSC 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 OSSC 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°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 μg 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°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 μm 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 β-actin.

*Fresh tissue*

Total DNA was extracted from fresh oral biopsies using the QIAamp tissue DNA Mini Kit manufacturer’s protocol (QIAgen, Hilden, Germany). Briefly, tissue samples were weighed, cut into small pieces, and incubated at 56°C by addition of 180 μL of ATL buffer supplied with 20 μL of proteinase K per 25 mg of sample. When tissues were completely lysed, a volume of 200 μL 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 μL 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 β-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 µL ten times PCR buffer, 0.6 µL ten times mix dNTP, 3.5 µL MgCl2 (25 mM), 0.3 µL oligonucleotide MY09 primer (100 mM), 0.3 µL oligonucleotide MY11 primer (100 mM), and 14.2 µL H2O to a final volume of 21.4 µL. In addition, 3.5 µL of the sample and 0.15 µL 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°C, 1 min at 55°C, and 2 min at 72°C. A final extension step at 72°C was carried out for 5 min, and then at 4°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 5×1 of PCR buffer, 2 mM MgCl2, 0.2 mM dNTP, 2 pmol of primer GP5+/GP6+, and 1 U of AmpliTaq. The thermocycler temperature programme consisted of denaturation at 95°C for 30 s, annealing at 45°C for 30 s, and extension at 72°C for 1 min for 35 cycles. Each PCR was initiated by a 5 min denaturation step at 95°C and finished by a 10 min extension step at 72°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 µL reaction volume, and the conditions were as follows: step of denaturation at 95°C for 30 sec, annealing at 52°C for 45 sec, and elongation at 72°C for 45 sec for 35 cycles, with an initial incubation at 95°C for 10 min and a step of final elongation at 72°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 µL 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 µL of the PCR product were analysed on 2% agarose gel (DNA Agar; Marine Bio Products Inc., Quincy, MA, USA) containing 0.5 gmol 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).

**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 OSSC 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 OSSC samples were found to be HPV DNA positive, using NPCR (Table 3). 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 OSSC cases, respectively (P<0.005) (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 OSSC cases, respectively. 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 3.

**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 OSSC, by highly sensitive PCR method. 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% [20-23]. High-risk HPV-16 has been clearly shown to be the dominant type in head and neck cancers [24-26]. 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 [27-29]. 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 [30-34].

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 [35].

In this study HPV-16 was detected in 82% and 94% of HPV-positive OED and OSSC samples, respectively. When these samples were analysed for integration, in 84% and 87% of HPV-16-positive OED and OSSC 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 [36]. 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 [37,38].

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 [39]. Many studies have shown that the nested PCR method is a sensitive and useful tool for HPV DNA detection compared with single PCR [40,41].

In conclusion our data indicate a rise in the HPV and HPV-16 detection in OED and OSCC. 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. 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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**Table 1.**The primer sequences used in the PCR reactions.

**Table 2.** Clinical parameters of the control, OED and OSSC oral lesions.

**Table 3.** Prevalence of HPV, HPV-16, integrated and episomal viral forms in control, OED and OSCC samples.