ORIGINAL ARTICLE

# Assessment of HPV-16 DNA in Oral Potentially Malignant Disorders Using PCR Technique

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## ABSTRACT

### Background

The role of human papillomavirus (HPV), particularly the high-risk type 16, in the pathogenesis of oral potentially malignant disorders (OPMDs) remains a subject of considerable debate. While its oncogenic potential in oropharyngeal carcinoma is well-established, its prevalence and significance in precursor lesions of the oral cavity are less clear.

### Methods

A case-control study was conducted on 100 participants: 60 patients with histopathologically confirmed OPMDs (20 each of OL, OSMF, and OLP) and 40 healthy individuals with normal oral mucosa as controls. Tissue biopsies were collected, and genomic DNA was extracted. PCR was performed using specific primers targeting the E6 oncogene region of HPV-16. The amplified products were visualized via agarose gel electrophoresis.

### Results

HPV-16 DNA was detected in 18.3% (11/60) of the OPMD cases, which was significantly higher than the 2.5% (1/40) positivity rate in the control group ($p = 0.019$). The highest prevalence was observed in OLP (25.0%, 5/20), followed by OL (20.0%, 4/20) and OSMF (10.0%, 2/20). A significant association was found between HPV-16 positivity and a history of tobacco chewing among OPMD patients ($p = 0.038$). The mean age of HPV-positive patients was slightly higher ($48.7 \pm 11.2$ years) than HPV-negative patients ($44.1 \pm 10.5$ years), though the difference was not statistically significant ($p = 0.18$).

### Conclusion

The study demonstrates a significant presence of HPV-16 DNA in a subset of OPMDs, particularly in patients with a history of tobacco use. This finding suggests that HPV-16 may act as a potential co-carcinogen in the multistep process of oral carcinogenesis. PCR-based detection of HPV-16 could serve as a valuable molecular tool for risk stratification and may aid in the surveillance of high-risk OPMDs.



## Keywords

Human papillomavirus, HPV-16, Oral potentially malignant disorders, Oral leukoplakia, Oral submucous fibrosis, Oral lichen planus, Polymerase chain reaction


## INTRODUCTION

Oral cancer represents a major global health burden, with oral squamous cell carcinoma (OSCC) accounting for over 90% of all oral malignancies [1]. A significant proportion of OSCCs develop from a spectrum of precursor lesions known as oral potentially malignant disorders (OPMDs), which include oral leukoplakia (OL), oral submucous fibrosis (OSMF), and oral lichen planus (OLP) [2]. The malignant transformation rates for these conditions vary, underscoring the need for a deeper understanding of their etiopathogenesis to identify reliable biomarkers for risk assessment.

While traditional risk factors such as tobacco, alcohol, and areca nut are well-established, the role of infectious agents, particularly human papillomavirus (HPV), has gained significant attention over the past few decades [3]. HPV is a small, double-stranded DNA virus with over 200 genotypes, of which 12-15 are classified as high-risk due to their association with various anogenital and head and neck cancers [4]. Among these, HPV-16 is the most oncogenic and is responsible for the majority of HPV-driven oropharyngeal squamous cell carcinomas [5].

The oncogenic potential of HPV-16 is primarily attributed to the activity of its E6 and E7


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oncoproteins, which bind to and inactivate the tumor suppressor proteins p53 and retinoblastoma (pRb), respectively. This interaction disrupts cell cycle regulation, inhibits apoptosis, and promotes genomic instability, thereby driving carcinogenesis [6].

Although the role of HPV-16 in oropharyngeal cancer is unequivocal, its involvement in lesions of the oral cavity proper and in OPMDs remains controversial. Numerous studies have reported widely varying prevalence rates of HPV DNA in OPMDs, ranging from as low as 0% to as high as 80% [7, 8]. This inconsistency can be attributed to differences in geographical location, sample types (fresh tissue vs. paraffin-embedded), detection methods (PCR vs. immunohistochemistry), and the specific HPV genotypes targeted.

Recent research has focused on the potential synergistic effect between HPV and traditional carcinogens like tobacco. It has been hypothesized that tobacco-induced mucosal damage may facilitate HPV infection and persistence, while HPV oncoproteins may exacerbate the mutagenic effects of tobacco carcinogens [9]. However, the nature of this interaction in the context of OPMDs requires further elucidation.

The polymerase chain reaction (PCR) technique, due to its high sensitivity and specificity, is considered the gold standard for the detection of HPV DNA in tissue samples [10]. It allows for the identification of even low copy numbers of viral DNA, providing a more accurate assessment of prevalence compared to other methods.

There is a clear research gap in understanding the precise prevalence of HPV-16 in different types of OPMDs within specific populations and its correlation with clinicopathological parameters. Such information is crucial for determining the utility of HPV-16 as a prognostic biomarker and for developing targeted surveillance strategies. Therefore, this study aimed to assess the prevalence of HPV-16 DNA in patients with OL, OSMF, and OLP using PCR and to correlate its presence with demographic factors, habit history, and clinical features.

## MATERIALS AND METHODS

### Study Design and Setting

This hospital-based case-control study was conducted over a period of two years, from January 2021 to December 2022..

Sample Size Calculation

Assuming an expected HPV-16 prevalence of 20% in OPMD cases and 5% in controls, with a 95% confidence level, 80% power, and a case-to-control ratio of 3:2, the minimum required sample size was calculated to be 52 cases and 35 controls. To enhance the statistical power and account for potential sample failures during processing, the final sample size was increased to 60 cases and 40 controls, totaling 100 participants.

Study Population

The study population was divided into two main groups:

- Case Group (n=60): Patients newly diagnosed with histopathologically confirmed OPMDs. This group was further subdivided into three equal subgroups of 20 patients each: Oral Leukoplakia (OL), Oral Submucous Fibrosis (OSMF), and Oral Lichen Planus (OLP).
- Control Group (n=40): Age- and gender-matched healthy individuals undergoing routine dental extractions, with no clinically evident oral mucosal lesions.

Inclusion and Exclusion Criteria

Inclusion Criteria for Case Group:

- Clinically and histopathologically confirmed diagnosis of OL, OSMF, or OLP.
- No prior history of treatment for the OPMD (surgical or medical).
- Willingness to undergo an incisional biopsy for research purposes.

Inclusion Criteria for Control Group:

- Clinically healthy oral mucosa with no history of OPMDs or oral cancer.
- No history of any systemic disease.

Exclusion Criteria (for both groups):

- Patients who had received any form of radiotherapy or chemotherapy in the head and neck region.
- Individuals with immunocompromised status (e.g., HIV infection, long-term steroid use).
- Presence of any other active oral infection or inflammatory condition.
- Recent use (within 1 month) of antiviral or immunomodulatory medications.

Tissue Collection and Processing

For the case group, an incisional biopsy (approximately 3x3 mm) was performed under local anesthesia from the most representative area of the lesion. For the control group, a small piece of healthy mucosal tissue was obtained during the extraction procedure. The tissue samples were immediately rinsed in sterile phosphate-buffered saline (PBS) and placed in sterile cryovials containing RNAlater™ Stabilization Solution (Thermo Fisher Scientific, USA). The samples were then transported on ice and stored at -80°C until DNA extraction.

DNA Extraction

Genomic DNA was extracted from the tissue samples using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Briefly, approximately 25 mg of tissue was mechanically homogenized, lysed with proteinase K, and the DNA was subsequently purified through a silica-based membrane. The concentration and purity of the extracted DNA were assessed using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific, USA). Samples with an A260/A280 ratio between 1.8 and 2.0 were considered pure and used for PCR analysis.

PCR Amplification

PCR was performed to amplify a 150-base pair fragment of the E6 oncogene of HPV-16. The reaction was carried out in a 25 µL total volume containing 12.5 µL of 2X Taq Master Mix (Thermo Fisher Scientific, USA), 0.5 µM each of forward and reverse HPV-16 E6-specific primers, 50 ng of template DNA, and nuclease-free water.

The primer sequences used were:

- Forward: 5'- GAG TAT GAG GAT TAT GCA TTT TTT G-3'
- Reverse: 5'- GCT GTT TCT GTT GTT GCT TGC T-3'

The PCR cycling conditions were as follows: initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds. A final extension step was performed at 72°C for 5 minutes.

Positive control (DNA from CaSki cells, known to harbor HPV-16) and negative control (nuclease-free water in place of template DNA) were included in each PCR run to ensure the validity of the results.

Detection and Analysis

The PCR products were separated by electrophoresis on a 2% agarose gel stained with ethidium bromide. The gel was visualized under ultraviolet light, and the presence of a 150 bp band was considered indicative of HPV-16 DNA positivity.

Statistical Analysis

The collected data were statistically analyzed using SPSS software version 22.0 (IBM Corp., Armonk, NY, USA). Descriptive statistics were used to summarize the data. The Chi-square test was used to compare the proportion of HPV-16 positivity between cases and controls and to assess its association with categorical variables. The independent samples t-test was used to compare the mean age between HPV-positive and HPV-negative groups. A p-value of less than 0.05 was considered statistically significant.

## RESULTS

Demographic and Habit Profile

The study included 100 participants, comprising 60 patients with OPMDs and 40 healthy controls. The demographic characteristics and habit history of the study population are detailed in Table 1. There was no statistically significant difference in the mean age or gender distribution between the case and control groups ($p > 0.05$), ensuring comparability. Among the OPMD patients, tobacco chewing was the most prevalent habit (55.0%), followed by smoking (28.3%) and alcohol consumption (15.0%).

**Table 1:** *Demographic characteristics and habit history of the study population*

| Parameter | OPMD Cases (n=60) | Healthy Controls (n=40) | p-value |
|---|---|---|---|
| Age (years, mean ± SD) | 45.2 ± 10.8 | 43.7 ± 9.5 | 0.421 |
| Gender (n, %) | | | 0.812 |
| Male | 38 (63.3%) | 24 (60.0%) | |
| Female | 22 (36.7%) | 16 (40.0%) | |
| Tobacco Chewing (n, %) | 33 (55.0%) | 5 (12.5%) | <0.001* |
| Smoking (n, %) | 17 (28.3%) | 8 (20.0%) | 0.352 |
| Alcohol Consumption (n, %) | 9 (15.0%) | 4 (10.0%) | 0.458 |

*p-value < 0.05 considered significant; OPMD: Oral Potentially Malignant Disorder

Prevalence of HPV-16 DNA

The prevalence of HPV-16 DNA in the study groups is summarized in Table 2. HPV-16 DNA was detected in 11 out of 60 OPMD cases, yielding an overall prevalence of 18.3%. In contrast, only 1 out of 40 control samples (2.5%) tested positive for HPV-16 DNA. This difference was statistically significant (p = 0.019). Among the OPMD subgroups, OLP showed the highest positivity rate (25.0%), followed by OL (20.0%) and OSMF (10.0%).

**Table 2:** *Prevalence of HPV-16 DNA in OPMD cases and controls*

| Group | Number of Samples | HPV-16 Positive (n, %) | p-value (vs. Controls) |
|---|---|---|---|
| OPMD Cases (Total) | 60 | 11 (18.3%) | 0.019* |
| Oral Leukoplakia (OL) | 20 | 4 (20.0%) | 0.048* |
| Oral Submucous Fibrosis (OSMF) | 20 | 2 (10.0%) | 0.241 |
| Oral Lichen Planus (OLP) | 20 | 5 (25.0%) | 0.017* |
| Healthy Controls | 40 | 1 (2.5%) | Reference |

*p-value < 0.05 considered significant; OPMD: Oral Potentially Malignant Disorder

Correlation of HPV-16 Positivity with Clinicopathological Factors

The correlation between HPV-16 positivity and various clinicopathological factors within the OPMD group is presented in Table 3. A statistically significant association was observed between HPV-16 positivity and a history of tobacco chewing (p = 0.038). Patients with tobacco-chewing habits were more likely to be HPV-16 positive. No significant correlation was found with age, gender, site of the lesion, or the presence of epithelial dysplasia.

**Table 3:** *Correlation of HPV-16 positivity with clinicopathological factors in OPMD patients (n=60)*

| Parameter | HPV-16 Positive (n=11) | HPV-16 Negative (n=49) | p-value |
|---|---|---|---|
| Age (years, mean ± SD) | 48.7 ± 11.2 | 44.1 ± 10.5 | 0.180 |
| Gender (n, %) | | | 0.775 |
| Male | 7 (63.6%) | 31 (63.3%) | |
| Female | 4 (36.4%) | 18 (36.7%) | |
| Tobacco Chewing (n, %) | | | 0.038* |
| Yes | 9 (81.8%) | 24 (49.0%) | |
| No | 2 (18.2%) | 25 (51.0%) | |
| Site of Lesion (n, %) | | | 0.612 |
| Buccal mucosa | 5 (45.5%) | 20 (40.8%) | |
| Tongue | 3 (27.3%) | 12 (24.5%) | |
| Others | 3 (27.3%) | 17 (34.7%) | |
| Epithelial Dysplasia (n, %) | | | 0.425 |
| Present | 4 (36.4%) | 12 (24.5%) | |
| Absent | 7 (63.6%) | 37 (75.5%) | |

*p-value < 0.05 considered significant

## DISCUSSION

The present study was designed to investigate the prevalence of HPV-16 DNA in a cohort of patients with different types of OPMDs using a sensitive PCR technique and to explore its potential association with various risk factors. The key finding was a significantly higher prevalence of HPV-16 DNA in OPMDs (18.3%) compared to healthy controls (2.5%), suggesting a potential role for this high-risk virus in the early stages of oral carcinogenesis.

The overall prevalence of 18.3% observed in our study falls within the wide range reported in the literature, which highlights the geographical and methodological variations in HPV detection [7, 8]. A meta-analysis by Miller and John [11] reported a pooled prevalence of HPV in oral leukoplakia of approximately 24.6%, which is comparable to our finding of 20.0% in the OL subgroup. The slightly higher prevalence in OLP

(25.0%) aligns with studies suggesting that OLP, particularly the erosive form, may be associated with viral infections [12]. The lower prevalence in OSMF (10.0%) is consistent with the hypothesis that the pathogenesis of OSMF is predominantly driven by areca nut-induced fibrosis, with potentially less contribution from viral factors [13].

The detection of HPV-16 DNA in 2.5% of our healthy control group is also consistent with the concept of asymptomatic oral carriage of HPV. It is estimated that a small percentage of the healthy population harbors HPV in their oral cavity, which may or may not progress to clinical disease [14]. The significant difference in prevalence between cases and controls, however, strengthens the argument for a pathogenic association rather than mere coincidence.

A particularly noteworthy finding from our study was the significant association between HPV-16 positivity and a history of tobacco chewing. This supports the theory of co-carcinogenesis, where traditional carcinogens and viral oncogenes act synergistically to promote malignant transformation [9]. Tobacco carcinogens can cause local immunosuppression and mucosal disruption, potentially creating a more favorable environment for HPV infection and persistence. Concurrently, the HPV E6 and E7 oncoproteins can interfere with DNA repair mechanisms, making the epithelial cells more susceptible to the mutagenic effects of tobacco-derived nitrosamines [15]. This synergistic relationship could accelerate the progression of OPMDs towards malignancy.

Interestingly, we did not find a significant correlation between HPV-16 positivity and the presence of epithelial dysplasia. This could be due to the relatively small sample size or the possibility that HPV infection may be an early event that precedes the development of full-blown dysplastic changes. It is also plausible that the oncogenic pathways driven by HPV are distinct from those that lead to histological dysplasia, a concept supported by studies showing that HPV-positive OSCCs often arise from dysplasia-free mucosa [16].

The high sensitivity of the PCR technique used in our study is a major strength, allowing for the detection of low copy numbers of viral DNA. However, this sensitivity also presents a limitation, as PCR cannot differentiate between active, transcriptionally active infection and the mere presence of viral DNA fragments. Future studies incorporating techniques like reverse transcription PCR (RT-PCR) to detect viral mRNA (E6/E7 transcripts) or p16 immunohistochemistry could provide more definitive evidence of oncogenic activity [17].

Another limitation is the cross-sectional design of our study, which provides a snapshot in time and cannot establish causality or determine the temporal sequence of events. A longitudinal follow-up study of HPV-positive versus HPV-negative OPMD patients would be invaluable in assessing whether HPV positivity confers a higher risk of malignant transformation.

## CONCLUSION

This study provides evidence for a significant presence of HPV-16 DNA in oral potentially malignant disorders, particularly in patients with a history of tobacco chewing. The findings suggest that HPV-16 may act as a co-carcinogen in the multistep process of oral carcinogenesis. The use of PCR-based detection of HPV-16 could serve as a valuable adjunctive tool for identifying a subset of high-risk OPMDs that may warrant more rigorous surveillance. Further large-scale, longitudinal studies are warranted to confirm these findings and to elucidate the precise role of HPV-16 in the progression of OPMDs to oral cancer.

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