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Detection of HPV16 in tissues of oral leukoplakia by polymerase chain reaction and p16 immunohistochemistry: A prospective study

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Abstract

Background: Globally, India accounts for highest incidence for oral cancer. Potential malignant disorders precede development of oral cancers. Leukoplakia is the one of the most common potentially malignant disorders and tobacco is a known risk factor for oral cancer. Role of viruses in oral carcinogenesis is not yet clear and its association with oral cancer has been a debated topic. The present study was aimed to evaluate the presence of human papillomavirus (HPV) in oral leukoplakia using polymerase chain reaction (PCR) and immunohistochemistry (IHC).

Methodology: Fifty clinicopathologically confirmed oral leukoplakia cases were included in the study. Detection of HPV was done using PCR and further validated with IHC.

Results: Among 50 subjects with oral leukoplakia, 43 specimens were reported as HPV positive through PCR and IHC confirmed p16 positivity in 5 specimens.

Conclusion: Study showed lower a prevalence of HPV in oral leukoplakia. Whenever in doubt, combined tests would often be beneficial for the detection of HPV.

Keywords

Human papillomavirus, immunohistochemistry, leukoplakia, polymerase chain reaction

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Introduction

In the Indian subcontinent, oral cancer accounts for 23% of all cancer-related deaths and the age-adjusted incidence rate of oral cancer in India is about 20 of 100,000.[1] Predominate oral cancers are oral squamous cell carcinoma (OSCC) accounting for over 90% of oral cancers.[2] Most of the oral cancers are preceded by potentially malignant disorders.[1,3] Leukoplakia is the most common potentially malignant disorder of the oral cavity.[4] The etiopathogenesis of leukoplakia is primarily due to tobacco usage or in combination with other confounding factors.[4,5] Leukoplakia may unpredictably regress, remain stable, or may progress to carcinoma. The presence of epithelial dysplasia is a marker for its malignant potential.[3,5]

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The specific role of human papillomavirus (HPV) in the development of leukoplakia and OSCC continues to be a debated topic. HPV and its role in the initiation of malignancy in the cervix is proven almost beyond doubt.[6] Recently, it has provoked lot of interest to elucidate the possible role of viruses in the initiation of oral carcinogenesis. Various studies[3-11] have suggested an association of HPV with OSCC and oral leukoplakia. In contrast, there are studies affirming a low prevalence of HPV in OSCC and oral leukoplakia.[11,12] Although there appears to be some link between HPV and oral leukoplakia, there is little evidence to support a causal relationship of any HPV infection and oral leukoplakia.[4]

Systematic reviews suggest that HPV is a risk factor for oropharyngeal cancers including posterior one-third (base) of the tongue and the tonsils. In contrast, HPV does not appear to be a significant risk factor for cancers of anterior two-thirds of tongue or remaining sub sites of the oral cavity.[13] In developing countries in south Asia like India, majority of these cancers are associated with tobacco or gutkha chewing.[14] The association of HPV in leukoplakia involving anterior parts of oral cavity is less researched. The aim of the study was to assess the prevalence of HPV in oral leukoplakia and its association with different sites of occurrence.

Materials and methods

This study was conducted in KLE Society's Institute of Dental Sciences, Bangalore, and Mazumdar Shaw Medical Center – Narayana Hrudayalaya, India. The Institutional Ethical Committees of both the centers approved the study and written informed consent was taken from all patients.

A total of 50 patients were included in the study within an age group of 18 to 73 years. A definitive diagnosis was done for white patch or plaque that cannot be characterized clinically or pathologically as any other disease and is not associated with any physical or chemical causative agent except use of tobacco.[15] Female patients, those with any history of HPV vaccination, other potentially malignant disorders, for example, oral submucous fibrosis and oral cancer were excluded. Participants in the study were interviewed for demographic data and information on tobacco usage.

The most representative areas with white or red and white lesions on oral mucosa were subjected to biopsy. The tissue specimens were stored in 10% formalin for histopathological evaluation to determine the degree of epithelial dysplasia. Another sample was collected, cut into 2 mm pieces and placed in 2 ml cryovials with RNAlater (three times the size of the tissue), stored overnight at 4°C, and then stored in –80°C. Following biopsy, the tissue specimens were microscopically examined for degree of epithelial dysplasia, according to the WHO grading system,[16] and evaluated for the presence of HPV through polymerase chain reaction (PCR).

HPV detection by PCR

DNA was extracted from 25 mg of biopsied tissue using the NucleoSpin nucleic acid extraction kit using the manufacturer's instructions (Macherey-Nagel, Denmark). Briefly, 180 µl of buffer T1 and 25 µl proteinase K solution were added to the microcentrifuge tube with the tissue specimen and then incubated in 56°C until complete lysis is obtained; 200 µl of buffer B3 was then added to the lysed solution and then incubated at 70°C for 10 min; 210 µl of ethanol was added to the sample and mixed vigorously. The sample was transferred to the NucleoSpin column and centrifuged for 1 min at 11,000 × *g*. The flow through was discarded and the column was placed back into the collection tube. The column was sequentially washed with buffer BW (500 µl) followed by buffer B5, and the column being centrifuged each time for 1 min at 11,000 × *g*. The final centrifugation was for 1 min at 11,000g to remove residual ethanol and transfered to 1.5 microcentrifuge tubes and DNA samples eluted with 100 µl of pre-warmed buffer BE were centrifuged for 1 min at 11,000 × *g* to obtain the DNA samples. The quality/quantity of DNA was determined by spectrophotometric analysis, and the quality of the sample was ascertained by amplification of housekeeping gene GAPDH (glyceraldehyde 3-phosphate dehydrogenase). Detection of HPV was carried out by HPV 16 specific primers (L1) using 200 ng of the DNA. A sample of cervical cancer was used as a positive control and a master mix without DNA of the sample was used as a negative control. The PCR reaction condition followed were initial denaturation at 94°C for 5 min followed by 40 cycles of denaturation at 94°C for 1 min. Then annealing at 55°C for 1-min extension at 72°C for 1 min followed by final extension at 72°C for 7 min. The products were run on 1% agarose gel and the specific sizes of the amplifications compared against molecular weight standards.

HPV detection by immunohistochemistry

The slides with tissue sections were kept at 37°C for overnight, de-paraffinized using xylene (5 min), and hydrated sequentially in 100% and 70% ethanol. The sections were then treated with 3% hydrogen peroxide in methanol for 10–30 min and washed in running water. Antigen retrieval was done using citrate buffer, the slides washed with Tris-buffered saline with Tween 20% (TBST) and incubated in blocking solution (DAKO) for 15 min. The primary antibody for p16 (sc-65224; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was diluted 1:10 and the sections were incubated overnight. The sections were then washed and subsequently treated with super enhancer for 30 min. Poly HRP was added for 30 min followed by TBST. DAB buffer was added as a substrate for color development, washed, and finally stained with hematoxylin. The slides were washed, dried, and then mounted. A slide of cervical cancer was used as a positive control. Slides, which had nuclear staining, were considered as positive for p16 by immunohistochemistry (IHC). The IHC score was calculated by multiplying the percentage of positive cells (0–100%) with the intensity (weak 1, moderate 2 and strong 3) to obtain a maximum score of 300.

Table 1. Detection of HPV in leukoplakia by PCR and IHC.

Total number of subjects with leukoplakia	HPV positive (%) by PCR	HPV negative (%) by PCR	HPV positive (%) by IHC	HPV negative (%) by IHC
50	43 (86%)	7 (14%)	5 (10%)	45 (90%)

HPV: human papillomavirus; PCR: polymerase chain reaction; IHC: immunohistochemistry.

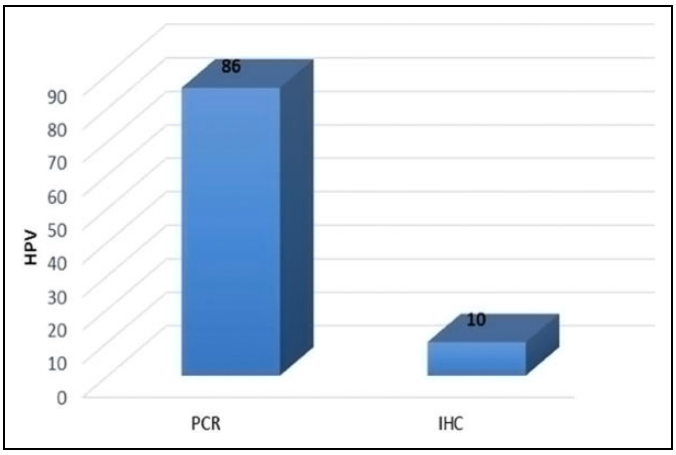

Figure 1. HPV positives in leukoplakia % through PCR and IHC. HPV: human papillomavirus; PCR: polymerase chain reaction; IHC: immunohistochemistry.

Results

The study group comprised of 50 clinicopathologically confirmed cases of leukoplakia, of which 38 were males and 12 females. In our study, the prevalence of oral leukoplakia was higher on buccal mucosa ($n = 46$) cases, followed by labial mucosa ($n = 2$), dorsal surface ($n = 1$), and on lateral border of tongue ($n = 1$).

Histologically grading of epithelial dysplasia showed mild ($n = 24$), moderate ($n = 11$), severe ($n = 10$), 4 showed epithelial alterations without any dysplasia and 1 had epithelial hyperplasia. PCR reported HPV positivity in 43 cases using L1 primer gene.

Further to validate our results, 50 samples were evaluated using IHC for the presence or absence of p16 staining. IHC reported only 5 HPV positives in the availed samples. Among the five positive patients, all the slides showed positivity in >10% of the cells with average IHC score being 123 $\pm$ 24. Among 43 PCR HPV positive cases, only five specimens were confirmed as HPV positive by IHC (Table 1 and Figure 1). Samples, which were HPV negative by PCR, were negative by IHC as well. Thus, IHC showed higher accuracy in determining HPV in leukoplakia.

The lesions on buccal mucosa showed higher frequency for the presence of HPV by IHC. Among different grades of epithelial dysplasia (Figure 2), 17% were HPV positive among 24 mild dysplastic lesions, 10% had HPV in 10 severe dysplastic lesions, while none were detected among moderate dysplasias.

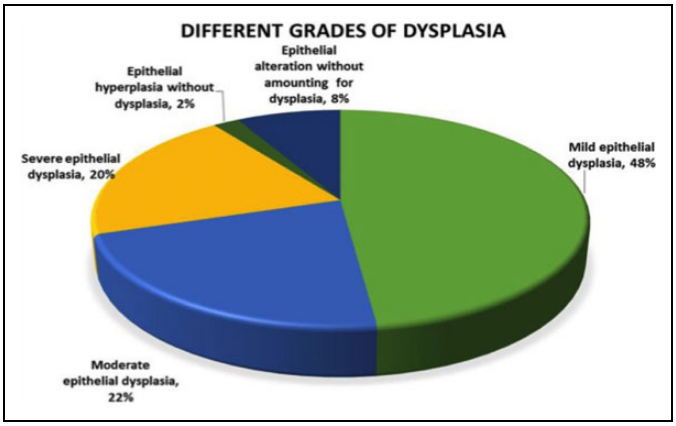

Figure 2. Degree of epithelial dysplasia in leukoplakia samples.

Discussion

HPV is epitheliotropic and infects either the cutaneous or mucosal epithelium, depending on their genotypes.[4] The genome of HPV consists of early region (E), late region (L), and long region control. Among early region, E5, E6, and E7 are oncoproteins.[5] L1 and L2 are structural proteins on viral capsid found in final stages of viral replication. There are low- and high-risk HPV genotypes that are associated with benign proliferative growths, precancerous, and cancerous epithelial lesions, respectively.[4,17-22]

HPV may act as an initiator of epithelial proliferation or play a role in the early stage of oral carcinogenesis involving E5, E6, and E7 oncoproteins.[5,22] These oncoproteins degrade oncosuppressor gene products: p53 and Rb.[4,5] There is enough evidence for oncogenic potential of high-risk genotypes in the pathogenesis of cervical cancer.[4] A correlation between HPV and OSCC exists because of the morphological similarities between the oropharyngeal and genital epithelia. Literature review shows prevalence of HPV in 20–50% of OSCC.[22,23]

The association of HPV in leukoplakia is highly controversial. It is extremely debatable whether HPV can be considered as an etiological or a malignancy risk factor in precancerous or cancerous oral lesions. In this context, some research groups identified high-risk HPV antigens and viral DNA in potentially malignant and malignant oral disorders. However, high frequencies of HPV were reported in healthy oral mucosa as well.[17]

Robinson et al.[24] described HPV infection can be demonstrated in fresh tissue by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) and was considered as gold standard as fresh samples contain intact mRNA. However, its utility is limited due to suboptimal preservation of routine biopsy samples that contain degraded RNA molecules. Hence, strategy for HPV testing in clinical practice for formalin-fixed paraffin-embedded tissue samples should be explored.

Previous studies demonstrate that PCR-based technique is a highly sensitive and specific method available for detecting HPV in the tissues.[22,23] Thus, our study intended to investigate the presence of type-specific HPV early proteins in oral

leukoplakia through PCR. However, early proteins in availed samples were unidentified. Detection of HPV early proteins is more complicated, as they are often expressed in low amounts in infected tissues.[25] The drawback in PCR techniques is false negative results due to multiple technical issues. In addition, the infections may be at lower copy numbers that are undetectable in the availed samples.[22] Hence, HPV genotypes present in the tissue specimen were not detectable with PCR using early gene-specific primers.

Another downside is that multiple infections, pathogenic and non-pathogenic, are not uncommon. Amplification of samples containing DNA from more than one HPV genotype can lead to a much stronger amplification of particular sequences present. This would complicate the detection of all genotypes in a sample with multiple infections. At times, additional, labor-intensive procedures, such as sequencing or type-specific PCR, are required. HPV will be detected often in unfixed fresh specimens than in formalin-fixed specimens, as there is denaturation of the DNA even after short-term fixation.[20]

Assessment by PCR using broad-spectrum consensus L1 capsid gene could detect HPV in oral mucosa in this study. It is produced in greater quantity during final stages of viral replication and preserved among different types of HPV. PCR reported 43 (86%) of HPV-positive lesions. However, a problem with the PCR-based detection is the possibility of false positivity, due to carry over of the product and contamination of paraffin-embedded tissue during routine processing. Although PCR can detect extremely small fragments of DNA, it may represent either the contamination of the sample or biologically insignificant HPV infection.[4] Routinely, such findings have been reported as pathologically significant. These could be legitimate findings or might be the results of inconsistencies and errors in methodology. This high level of false positivity could be the probable cause for very high association of HPV in leukoplakia by PCR in this study.

Several studies have also shown the association of HPV in oral leukoplakia.[13,14,23] Cianfriglia et al. revealed the presence of HPV in various types of Leukoplakia and described that HPV may be a cofactor in carcinogenesis. A study by Cianfriglia[11] reported the highest incidence of HPV (about 60%) in leukoplakic lesions in the reported literature and avowed that HPV as a specific risk factor in premalignant disorders.[11] Study by Sugiyama et al.[12] have showed high prevalence of HPV types 16 and 18 in leukoplakia. A study by Mathew et al.[6] has observed HPV 18 to have higher prevalence in leukoplakia. A meta-analysis on HPV in oral carcinoma and OPMDs revealed a potentially important causal association between HPV and OSCC and in OPMDs such as oral leukoplakia, oral lichen planus, and epithelial dysplasias.[26]

As there was ambiguity and high prevalence of HPV through PCR, the results were validated with IHC. IHC staining for expression of p16 protein was used as a surrogate marker for HPV detection alternative to PCR. In our study, only 5% showed positive results for HPV in leukoplakia samples by IHC. Schache et al.[27] showed the suboptimal analytical performance of p16 IHC as a surrogate marker for HPV infection indicating p16 IHC lacks specificity. The authors highlighted the detection of HPV status with the use of single lab test would be limited and concluded the possibility of combined tests would approach the gold standard. It has been shown that other assays such as PCR for HPV DNA, HPV16/18 fluorescent in situ hybridization (FISH) and p16 IHC have suboptimal performance when used as stand-alone tests, with FISH being the gold standard. Hence in this study, a combination of PCR and IHC was used to ascertain positivity.

In India, the most common site of occurrence of leukoplakia is buccal mucosa, followed by labial mucosa, lateral and dorsal surfaces of tongue, and the floor of the mouth. The OPMDs on the tongue and floor of the mouth are more prone for malignant transformation.[5] In our study, leukoplakia was more predominant on the buccal mucosa due to specific gutkha chewing habits in this population.

Epithelial dysplasia in oral leukoplakia is a useful marker of the risk of carcinomatous transformation and is an important guide in clinical management.[4] The reported prevalence of epithelial dysplasia in oral leukoplakia ranges from 5% to 25%. Dysplasia is more frequent in nonhomogeneous than in homogeneous leukoplakia, and it is probable that dysplasia is the histopathological expression of genomic and molecular alterations in a field of keratinocytes.[18,19] The incidence of HPV increases from normal mucosa to dysplasia and to cancer.[20] Leukoplakia associated with moderate and severe dysplasia are more prone for malignant transformation.[4,18-20]

Forty-five samples exhibited grades of epithelial dysplasia, among these, HPV was identified in four mild epithelial dysplasia and in one severe dysplastic lesion by IHC. However, we could not correlate HPV with severity of dysplasia that was in accordance with study conducted by Jayaprakash et al.[21] Five cases that were positive for HPV were under rigid follow-up since 1.5 years and clinically have not shown any progression to malignancy.

This study adopted L1-based PCR assays and p16 IHC for ascertaining the HPV positivity in oral leukoplakia. Further to reports that suggest a wide range of HPV association with oral cancer, these multiple assays were adopted. Application of gold standard, in situ hybridization for HPV detection was not undertaken due to limited resources. However, this study also did not undertake HPV typing by PCR, which remains an important limitation of this research. In addition, IHC by E6/E7 proteins of HPV might also provide insights into the potency of the HPV infections in the patients. Further studies with larger cohorts of patients that account for site/stage-based differences of oral mucosa might also enable a correlation of HPV16/p16 detection/expression with grade of dysplasia.

Conclusion

Our study showed lower prevalence of HPV in oral leukoplakia. Whenever in doubt, combined tests would often be beneficial in detection of HPV.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Translational value

Prevalence of HPV in oral leukoplakia was found to low. Hence the role of HPV in Oral Leukoplakia in our settings was found to be minimal.