Prevalence of Human Papillomavirus (HPV) 16 and 18 in Oral Malignant and Potentially Malignant Disorders: A Polymerase Chain Reaction Analysis – A Comparative Study

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Abstract

Introduction: Human papillomavirus (HPV) are now being increasingly associated as a cause of oral squamous cell carcinomas (OSCC). This study was designed to evaluate the prevalence of HPV in Pelizaeus-Merzbacher disease (PMD) and OSCC using polymerase chain reaction that might help in better understanding of the role played by this virus in the oncogenic process even from its evolution stage. **Materials and Methods:** Formalin-fixed paraffin-embedded tissue samples ($n = 40$) of OSCC and mild, moderate, and severe dysplasia were used for this study. DNA was quantified and checked for purity spectrophotometrically. Statistical analysis was performed using SPSS software and statistical significance was assessed using Fischer's exact test ($p < 0.05$ was considered significant). **Results:** High-risk (HR)-HPV-16 was found to be positive in 35% of OSCC cases which showed a statistically significant association of HPV 16 with OSCC. Verrucous carcinoma had predominant HPV 16 infection (60%), followed by SCC with 40%. However, this association was not statistically significant. None of the OSCC samples were infected with HPV 18. Among the PMD, we found only 5% showing HR-HPV 16 infection which was not significant. **Discussion:** Although OSCC is attributed to tobacco and alcohol consumption, a significant proportion of OSCC cases have been demonstrated to contain HPV types. The high-risk HPV type 16 tends to be the most predominant type detected in cases of OSCC.

Keywords: Human papillomavirus 16, human papillomavirus 18, oral cancer, oral squamous cell carcinomas, polymerase chain reaction, potentially malignant disorders

Introduction

Oral malignancy is a major global health problem and it constitutes the sixth-most common malignancy.[1] More than 90% of these malignancies representing squamous cell carcinoma (SCC), in the oral cavity are often preceded by preexisting oral lesions termed as potentially malignant disorders of the oral mucosa.[2] Oral SCC (OSCC) are characterized by a multiphasic and multifactorial etiopathogenesis. In India and other regions of Southeast Asia, it is the predominant malignancy,[3] accounting for up to 50% of all malignant tumours. Along with tobacco and alcohol, factors such as genetic predisposition, diet, and viral agents like human papillomavirus (HPV),[4] may also play a role in the initiation or development of oral carcinogenesis.

Approximately 15% of all malignancies worldwide appear to be connected with viral infections and several human DNA viruses are now accepted as causative factors.[5] Majority of the head-and-neck malignancies originate from the epithelium which line the upper aerodigestive tract.[6]

The epithelial areas of the upper aerodigestive tract display greatest susceptibility to HPV due to the exposure of the basal cells to HPV infection.[7] Among the strains, HPV-16

Access this article online

Quick Response Code:

Website: www.amsjournal.com

DOI: 10.4103/ams.ams_376_20

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Received: 06-02-2021 **Last Revised:** 01-03-2021
Accepted: 15-03-2021 **Published:** 24-07-2021

How to cite this article: Sri S, Ramani P, Premkumar P, Ramshankar V, Ramasubramanian A, Krishnan RP. Prevalence of Human Papillomavirus (HPV) 16 and 18 in oral malignant and potentially malignant disorders: A polymerase chain reaction analysis – A comparative study. Ann Maxillofac Surg 2021;11:6-11.

and -18 are the major high-risk (HR) types and predominate in OSCC.[8]

HPV-associated carcinogenesis is mediated by expression of the viral E6 and E7 oncoproteins,[9] which cause deregulation of the cell cycle by inactivating p53 and pRb, respectively. The E6 protein induces degradation of p53 through ubiquitin-mediated proteolysis,[10] leading to substantial loss of p53 activity. The usual function of p53 is to arrest cells in G1 or induce apoptosis, to allow host DNA to be repaired. E6-expressing cells are not capable of this P53-mediated response to DNA damage and hence, they are susceptible to genomic instability. The E7 protein binds and inactivates the retinoblastoma tumour suppressor gene product pRB, causing the cell to enter S-phase, leading to cell-cycle disruption, proliferation, and malignant transformation.[11]

HPV are now being increasingly associated with the cause of OSCC.[12] Compared with their HPV-unrelated counterparts, HPV-related HNSCCs tend to occur in younger patients[13] who do not smoke or drink alcohol[14] localize to the oropharynx**,** and are highly curable even when presenting as locoregionally advanced disease.[15,16] HPV status has a considerable effect on patient profiles and clinical outcomes.

In general, oral epithelium undergoes a sequence of histopathological premalignant transformations before the development of invasive carcinoma.[3] The detection of HPV in Pelizaeus-Merzbacher disease (PMD) would be essential to recognize the factors that determine its capability to induce malignant transformation in OSCC. An increasing prevalence of HPV in PMD suggests that it may play a role in malignant transformation. Hence, studying the prevalence of HPV detection in oral lesions associated with the dysplastic progression of epithelium may help to define the role of this virus in the development of OSCC.

There is a greater risk of carcinomatous transformation of idiopathic leukoplakia, nonhomogenous leukoplakia, leukoplakia affecting the HR sites, leukoplakia with high-grade epithelial dysplasia, and of leukoplakia, in which the keratinocytes carry cytogenetic alterations associated with carcinomatous transformation.[17] Although there appears to be some link between HPV and oral leukoplakia, there is little evidence to support a causal relationship either between HPV infection and oral leukoplakia or between HPV-infected keratinocytes and their malignant transformation.

HPV may play either an oncogenic or a co-oncogenic role in some HPV-infected precancerous and cancerous epithelial neoplasms.[18] HPV may be risk factor for leukoplakia, and is responsible for the progression to oral malignancy.

Oral submucous fibrosis (OSMF) is a chronic potentially malignant disorder, a malignant transformation rate[19] as high as 7.6% have been reported from the Indian subcontinent over 17 years. Areca nut is the proven causative factor.[20] However, other factors, including viruses, especially HPV, may also play a role in the initiation or development of malignancy in these lesions. At present, there is a paucity of information on the potential role of HPV in potentially malignant oral lesions.

Hence, this study was designed to evaluate the prevalence of HPV in PMD and OSCC using polymerase chain reaction (PCR) that might help in better understanding of the role played by this virus in the oncogenic process even from its evolution stage. Investigation on the role of HPV could be rewarding in planning long-term strategies for prevention, diagnosis, and possible treatment option of these (leukoplakia, OSMF, and OSCC) conditions.

Materials and Methods

This cross-sectional comparative study was conducted in the Department of Oral and Maxillofacial Pathology. This study was approved by the Institutional ethical committee and the study conforms to the recognized standards required by Declaration of Helsinki. Formalin-fixed paraffin-embedded tissue samples (n = 40) was used for this study. Group I comprised OSCC (n = 20) cases which were further classified into four categories according to the histological type and grade namely (verrucous carcinoma n = 5), well differentiated SCC (n = 5), moderately differentiated SCC (n = 5), poorly differentiated SCC (n = 5). Group II comprised PMD (n = 20), of which 10 cases were clinically leukoplakia and histopathologically hyperkeratosis with mild (n = 3), moderate (n = 5), and severe dysplasia (n = 2) and 10 cases of OSMF with histological grading of moderately advanced (n = 3) and advanced (n = 7) were retrieved from the archives of the Department of Oral and Maxillofacial Pathology from March 2010 to March 2012.

Demographic data, including age at diagnosis, sex, and habits (smoking, alcohol, and tobacco exposures) were obtained from the clinical record. Tumour site, size, type of biopsy, and histopathological types were determined from operative and pathology notes. A 2 member panel of certified Oral and Maxillofacial Pathologists re-verified all the samples the histologic diagnosis of OSCC and PMD, namely different grades of hyperkeratosis with dysplasia (clinically leukoplakia) and OSMF. A consensus diagnosis was reached in all the cases after examination of multiple hematoxylin- and eosin-stained sections.

DNA extraction and diagnosis of high-risk human papillomavirus infection by polymerase chain reaction

DNA was extracted from formalin-fixed paraffin embedded tissues samples by using the Qiagen QIAamp DNA tissue Kit (Qiagen Inc., USA). The extracted genomic DNA was quantified and checked for purity spectrophotometrically (Spectro UV-Vis Double Beam PC). Ethidium bromide stained 1.2% agrose gel electrophoresis was used to confirm presence of DNA in samples.

β-globin gene primer was examined for the DNA quality control in all specimens, FP 5’ACACAACTGTGTTCACTAGC 3’ and RP 5’ CAACTTCATCCACGTTCACC 3’, with an amplicon

size 110 bp. The samples with β-globin positivity were taken for further analysis for HPV by PCR.

We used GP5+/GP6 + primers for PCR-based HPV detection. The sequences are shown in the Table 1. GP5+ (FP) 5' TTTGTTACTGTGGTAGATACCAC 3'/GP6+ (RP) 5'GAATATGATTTGCAGTTTATTTTTC 3' primers were selected because of the short amplicon size and also because it can detect >15 mucosal types of HPV.

HPV subtype 16 and 18 were identified by type-specific PCR.

Further typing of high-risk HPV types 16 and18 was done by type-specific HPV 16 (FP) 5'-TGCTAGTGCTTATGCAGCAA-3' and (RP) 5' ATTTACTGCAACATTGGTAC-3' and HPV18 (FP) 5'-AAGGATGCTGCACCGGCTGA-3' and (RP) 5'-CACGCACACGCTTGGCAGGT-3' with an amplicon size 152 bp. SiHa served as positive controls for HPV 16, HeLa for HPV 18.

PCR was performed in reaction mixture containing 2 µL-10X PCR Buffer, 1.2 µL-1.5 mM MgCl2, 0.5 µL-5 mM dNTPs, 12.3 µL-Milli Q, 0.8 µL-5 pM/λ F Primer, 0.8 µL-5 pM/λ R Primer, 0.4 µL-Taq Polymerase, 2 µL-DNA. The thermal cycling condition was initial denaturation at 95°C for 10 min, denaturation at 94°C for 35 s, annealing at 55°C for 1 min and extension at 72°C for 40 s, X40 cycles, final elongation 72°C for 8 min, hold 4°C.

Statistical analysis

Statistical analysis was performed using the SPSS 17.0 (IBM SPSS Statistics 27.0) (Statistical Package for Social Sciences) statistical package. Fischers exact Chi-square test were used in this study. $P < 0.05$ was considered significant and $P > 0.05$ was considered as not significant.

Results

Prevalence of human papillomavirus 16 and 18 genotypes in Group I – OSCC

HR-HPV-16 was found to be positive in 35% (7/20) of OSCC cases which showed a statistically significant association of HPV 16 with OSCC. Association of HPV 16 in subgroups of OSCC is shown in Table 2. Verrucous carcinoma had predominant HPV 16 infection (60%, followed by SCC with 40%. However, this association was not statistically significant. None of the OSCC samples were infected with HPV 18. PCR analysis of HPV 16 DNA in well-differentiated SCC and Verrucous carcinoma is given in Figure 1.

Prevalence of HPV 16 and 18 genotypes in Group II – Potentially malignant disorder: (hyperkeratosis with epithelial dysplasia (clinically leukoplakia) and different grade of OSMF).

Among the potentially malignant disorders, we found only 5% (1/20) showing HR-HPV 16 infection which was not significant. This case was identified as hyperkeratosis with severe dysplasia. There was no HPV 18 infection identified in this group. None of the OSMF cases showed HPV 16 and 18 infection by diagnosed by PCR.

Table 1: Primers used for the study

Primer	Sequence
GP5+ /GP6+ PRIMERS	
GP5+ FORWARD	5' TTTGTTACTGTGGTAGATACCAC 3'
GP6+ REVERSE	5'GAATATGATTTGCAGTTTATTTTTC 3'
HPV 16 PRIMERS	
HPV 16.L1 FORWARD	5' TGCTAGTGCTTATGCAGCAA 3'
HPV 16.L1 REVERSE	5' ATTTACTGCAACATTGGTAC 3'
HPV 18 PRIMERS	
HPV 18 FORWARD	5' AAGGATGCTGCACCGGCTGA 3'
HPV 18 REVERSE	5' CACGCACACGCTTGGCAGGT 3'

HPV=Human papillomavirus

Table 2: Human papillomavirus 16 positivity in Group I and Group II - sub groups

Groups	Histopathological type	Frequency *n* (%)
Group-I	Verrucous carcinoma	
	HPV 16 positivity	
	Negative	2 (40.0)
	Positive	3 (60.0)
	Total	5 (100.0)
	Well-differentiated OSCC	
	HPV 16 positivity	
	Negative	3 (60.0)
	Positive	2 (40.0)
	Total	5 (100.0)
	Moderately differentiated OSCC	
	HPV 16 positivity	
	Negative	4 (80.0)
	Positive	1 (20.0)
	Total	5 (100.0)
	Poorly differentiated OSCC	
	HPV 16 positivity	
	Negative	4 (80.0)
	Positive	1 (20.0)
	Total	5 (100.0)
Group-II	Hyperkeratosis with mild dysplasia	
	HPV 16 positivity	
	Negative	9 (90.0)
	Positive	1 (10.0)
	Total	10 (100.0)
	Oral submucous fibrosis (Grade III and IV)	
	HPV 16 positivity	
	Negative	10 (100.0)
	Total	10 (100.0)

HPV=Human papillomavirus; OSCC=Oral squamous cell carcinomas

Comparison of prevalence of human papillomavirus 16 and 18 between the groups

The difference in positivity of HPV 16 and 18 between

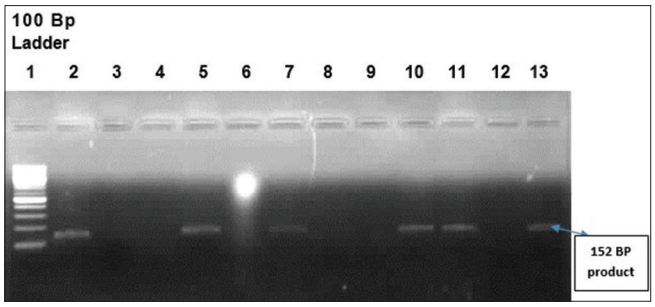

Figure 1: Polymerase chain reaction analysis of human papillomavirus 16 DNA in cases of Group–I (well-differentiated squamous cell carcinomas and Verrucous carcinoma). Lane 1, 100 bp Ladder; Lane 2, positive control from SiHa cells, Lane 3: Negative control of human papillomavirus-DNA, Lane 5, 7, 10, 11, 13 show positive samples for human papillomavirus 16 DNA

cases of OSCC, potentially malignant disorders were compared using the Fischers exact Chi-square test, there was a significant difference in HPV 16 infection between the Groups ($P < 0.044$). HPV-18 was not detected in any of the groups. Subgroup analysis could not be done as the subgroups in Group I and Group II were different from each other.

Comparison between human papillomavirus 16 and human papillomavirus 18 prevalence

HPV 16 positivity was found to be 20% in Group I and Group II. Group I showed 35% HPV 16 infection among OSCC, and 5% in the Group II cases of potentially malignant disorders, especially in hyperkeratosis with sever dysplasia. HPV-18 was negative in the entire group.

Relationship between the presence of HPV 16 and 18 genome and clinicopathological variables of Group I and Group II (combined $n = 40$).

HR-HPV 16 infection among the OSCC cases in Group I had a significant association with patient age in younger group (21–40 years) ($P < 0.030$), gender = males ($P < 0.046$) and site-oral tongue ($P < 0.024$). However, there was no association with the habits. Majority (85%) of the patients in this study had tobacco and alcohol-related habits (34/40). Interestingly, among the remaining 6 patients with no habits, 50% (3/6) were found to be HPV 16 positive. Among the 34 patients with habits, only 14.7% (5/34) were HPV 16 positive. However, this association was not found significant statistically. These associations were not found significant among the potential malignant disorders in Group II.

Discussion

HPV are reported principally in the ano-genital tract, oro pharyngeal region, and oral mucosa. HPV, especially the high-risk (HR) types (16 and 18), which are widely recognized in the promotion of tumourigenesis in female uterine cervical SCC,[21,22] also known to play an important role in OSCC.[23,24]

The concept of a two-step process of cancer development in the oral mucosa, i.e., the initial presence of a precursor lesion (potentially malignant disorder) subsequently developing into cancer, is well-established. Oral leukoplakia and OSMF are the high risk potentially malignant disorder. The detection of HPV in PMD would be essential to recognize the factors that determine its capability to induce malignant transformation, especially in OSCC.

In the present study, 20% of the cases showed HPV positivity and 80% did not detect any HPV among Group I OSCC and Group II PMD cases. The HPV involvement in head and neck carcinogenesis was first proposed in 1983 by Syrjanen *et al*. There is an extreme variation in the reported prevalence of HPV infection in OSCC and PMD ranging from 0% to 100%.[25] This is owing to differences in sampling and HPV detection methods, differences in ethnicity, geographic locations, and sample size and to the inappropriate grouping together of different lesions from different anatomical locations. It is clear that that the nonstandardisation of these multiple variables has not brought clarity to the field.

The prevalence of HPV 16 in Group I OSCC cases was 35%. This finding is consistent with the previous studies who have reported positivity range as 35% in Sugiyama,[26] (2003), 34% Zhang,[27] (2004), 36% Koppikar,[28] (2005), respectively. Though OSCC is attributed to tobacco and alcohol Consumption, a significant proportion of OSCC cases have been demonstrated to contain HPV types. The high-risk HPV type 16 tends to be the most predominant type detected in cases of OSCC.

A "hit and run" theory has been proposed to explain the HPV involvement in virus negative tumours which could develop from HPV-containing precursors, not requiring the HPV to maintain the malignant state, according to which viral genome does not need to be present to maintain cell transformation once genetic damage has been inflicted at an early stage.[29,30]

HPV 16 positivity among the subgroup of OSCC showed 60% positivity in verrucous carcinoma, 40% for well-differentiated SCC, 20% in moderately differentiated SCC, and 20% in poorly differentiated SCC, respectively. Verrucous carcinoma is a locally invasive and nonmetastatic variant of OSCC. The result of our study is consistent with previous data; Gonzalez *et al*. reported that the level of HPV16 is markedly higher in verrucous carcinoma (88.9%) than in conventional SCC (43.8%).[31]

Pintos *et al*., Tsuhako *et al*. reported that HPV-positive OSCCs usually have a good degree of differentiation. It is not known whether this is because of HPV induces changes in epithelial cells that lead to their differentiation to neoplastic cells with the ability to form keratin (well differentiated), or because this type of virus has a tropism for keratinized cells.[32,33] These reports are consistent with our result in which HPV detection was seen in 60% of verrucous carcinoma, followed by 40%, in well-differentiated SCC which are lesions showed more keratinisation. This substantiates that these carcinomas have a better prognosis in comparison with other grades and types of OSCC. However, in the present study, the positivity of HPV

16 was not statistically significant when correlated with the histological subgroups of OSCC which is in agreement with previous reports of Woods e*t al*.[34] as well as Holladay and Gerald.[35]

In our study, 5% of potentially malignant disorder cases showed positivity for HPV 16, especially in cases of hyperkeratosis with severe dysplasia (clinically leukoplakia), though the positivity was not statistically significant. HPV involvement in the etiology of potentially malignant lesions (leukoplakia) has been proposed by some authors.[35-38] Our finding of a 5% HPV 16 detection rate is consistent with the HPV 16 prevalence reported for such lesions in other PCR based studies.[35-38]

In a meta-analysis of HPV as a risk factor for OSCC, HPV detected in normal oral mucosa (10%) was significantly less than that in benign leukoplakia (22.2%) and OSCC (46.5%) and the probability of detecting HPV in oral carcinoma was approximately 4.7 times higher than in normal oral mucosa and HPV positivity progressively increase from dysplastic lesion (potentially malignant disorder) to OSCC.[24] This is consistent with our study findings where HPV positivity was seen in 10% cases of hyperkeratosis with severe dysplasia. Sugiyama *et al*. detected HPV-16 and HPV-18 DNA in normal, dysplastic, and malignant oral epithelium and strongly suggested that the prevalence of HPV16 is higher in dysplasia progressing to OSCC.[26] Hence, HPV-16 may be involved in the early stages of the development of OSCC arising from hyperkeratosis with dysplasia. This may also explain in a small way, the variation in the transformation rates hyperkeratosis with different grades of dysplasia.[39]

In the present study, HPV 16 and 18 was not detected in different grades of OSMF. This finding is consistent with another study that reveals a very low rate of HPV positivity in OSMF[1] though few authors have detected HPV 16 in OSMF.[38] However, since arecanut chewing has been proved to be the causative factor, viruses, especially HPV may have only a minimal role in the pathogenesis and malignant transformation of OSMF apart from the fact that HPV exhibits epitheliotropism.

HPV18, the second-most common type was found much less frequently in OSCC than adenocarcinomas of the cervix and in fact was undetected in all our groups. We had explored HPV 18 as glandular tissue is also a type epithelial tissue and whether it had any occurrence in oral mucosa. HPV18 has a special tropism for glandular tissue and is the most frequently detected type in adenocarcinomas of the cervix. Adenocarcinomas are rare in the head and neck and occur mainly in salivary gland tumours, which were not included in this study. HPV18 seems to be less effective at evading the host immune response and is less likely to persist compared with HPV16.

In our study, HPV 16 was found to be positive OSCC presenting among younger age group (21–44 years). Interestingly, 50% of the OSCC patients with no habits had HR HPV infection. This indicates that patients with younger age at presentation and with no habits should be diagnosed for HR-HPV infection.

Limitations of the present study are that the samples were taken from paraffin-embedded blocks rather than fresh tissue and more sophisticated molecular techniques may have increased the chance of detecting low copies of virus. Larger studies are needed to obtain a more precise estimate of the prevalence and persistence of HPV in OSCC and potentially malignant disorders to better understand the etiopathogenesis of HPV-related carcinogenesis. There is wide heterogeneity and socio-demographic variance in HPV prevalence rate in different geographical population and the need for quantification of different vaccination policies. HPV 18 may not have any role in the OSCC and potentially malignant disorders as seen in our study due to its affinity to the glandular tissue.

Conclusion

Interestingly, HPV positive tumours are biologically distinct from HPV negative tumours, the detection of HPV in OSCC is a favorable prognostic marker. HPV detection is recognized as a valid method to discern the presence and progress of disease encompassing all aspects of patient care including early cancer detection, prediction of therapeutic response, and selection of therapeutic strategies, tumour surveillance, and determination of tumour origin.

Accordingly, inclusion of HPV status as a component of emerging molecular staging systems is compelling, and routine HPV assessment will soon become part of the standard pathologic evaluation of all OSCC. However, in cases of potentially malignant disorders, high-risk HPV's role is yet to be defined, though as seen in our study may indicate their malignant potential.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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