# Human Papilloma Virus in Oral Squamous Cell Carcinoma – The Enigma Unravelled

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*Squamous cell carcinoma of the head and neck (HNSCC) has long been regarded as a disease entity having a remarkable incidence worldwide and a fairly onerous prognosis; thus encouraging further research on factors that might modify disease outcome. Squamous cell carcinomas encompass at least 90% of all oral malignancies. Several factors like tobacco and tobacco-related products, alcohol, genetic predisposition and hormonal factors are suspected as possible causative factors. Human papilloma virus (HPV), the causal agent of cervical cancer also appears to be involved in the aetiology of oral and oropharyngeal cancer. HPV-positive squamous cell carcinoma (SCC) seems to differ from HPV-negative SCC. Many questions about the natural history of oral HPV infection remain under investigation. The aim of this review is to highlight the current understanding of HPV-associated oral cancer with an emphasis on its prognosis, detection and management.*
**Key words:** *HPV, Koilocytes, Squamous cell carcinoma*

Cancer is one of the most common causes of morbidity and mortality today, with more than 10 million new cases and more than 6 million deaths each year worldwide. Oral cancer accounts for 2% to 4% of all cancer cases. Oral cancer is the predominant malignancy in India and other regions of Southeast Asia, accounting for up to 50% of all cancer[1]. Oral cancer includes a group of neoplasms affecting the oral cavity, pharyngeal regions and salivary glands. However, this term tends to be used interchangeably with oral squamous cell carcinoma (OSCC), which represents the most frequent of all oral neoplasms. It is estimated that more than 90% of all oral neoplasms are OSCCs[2]. OSCC has been found to be associated with tobacco, alcohol abuse and betel nut chewing; however, recent studies suggest that viral factors, including human papillomaviruses (HPV), may contribute to the aetiology of this malignant neoplasm[3,4]. High-risk HPV (types 16, 18, 31 and 33) have been known to affect epithelial cells by expressing proteins that alter tumour suppressor pathways by inactivating p53 and the retinoblastoma gene (Rb)[5]. Recently, HPV type 16 has been identified in 90% of HPV-associated head and neck tumours and has been found in 50% of oropharyngeal head and neck squamous cell carcinomas. However, markedly varied estimates of HPV prevalence in premalignant head and neck lesions (0 to 100%) have made it difficult to clarify the timing and nature of the contribution of HPV to head and neck carcinogenesis[6,7]. Since the first evidence of HPV in the aetiology of OSCC was shown in 1977, numerous studies have been conducted supporting the role of HPV as a causative agent for OSCC[8]. The aim of this review is to highlight the current understanding of HPV-associated oral cancer with an emphasis on its prognosis, detection and management.

**What is HPV?**

HPVs are small, epitheliotropic, nonenveloped, double-stranded DNA viruses that belong to the family papillomaviridae[9]. HPVs are called papilloma viruses because some of the HPV types cause warts or papillomas, which are non-cancerous tumours. But some types of HPV are known for causing cancer, especially of the cervix (the base of the womb at the top of the vagina). HPVs are

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**Table 1**  HPV genotypes and their associated diseases.

| Diseases and anatomical sites | HPV genotype |
|---|---|
| 1. Benign oral lesions | |
| 1.1 Oral squamous cell papilloma | HPV types 6 and 11 |
| 1.2 Veruca vulgaris (common wart) | HPV types 1, 2, 4, 7 and 57 |
| 1.3 Condylomata acuminata | HPV types 2, 6, 11 |
| 1.4 Focal epithelial hyperplasia (Heck's disease) | HPV types 13 and 32 |
| 2. Potentially malignant oral lesions | |
| 2.1 Leukoplakia | HPV types 6, 16 and 18 |
| 2.2 Erythroplakia | HPV types 6, 11, 18, 31 and 33 |
| 3. Oral and oropharyngeal squamous cell carcinoma | HPV types 16 and 18 |
| 4. Recurrent respiratory papillomatosis | HPV types 6 and 11 |
| 5. Anogenital | |
| 5.1 Condylomata acuminata | HPV types 6 and 11 |
| 5.2 Intraepithelial neoplasia | |
| 5.2.1 Low-grade | HPV types 6 and 11 |
| 5.2.2 High-grade | HPV types 16 and 18 |
| 5.3 Squamous cell carcinoma | HPV types 16 and 18 |
| 6. Cutaneous | |
| 6.1 Common warts | HPV types 1 and 2 |
| 6.2 Flat warts | HPV types 3 and 10 |

epitheliotropic oncogenic DNA viruses with more than 120 identified genotypes: the so-called high risk (HR) HPV, such as HPV-16 and HPV-18, have been definitively recognised as being strongly associated with cervical cancers[9]. There are more than 150 known strains, of which about 3 out of 4 (75%) HPV types are called cutaneous because they cause warts on the skin and the other 25% of the HPV types are considered mucosal types of HPV[10].Table 1 shows HPV genotypes and their associated diseases.

The involvement of human papillomaviruses (HPV) in head and neck carcinogenesis was first suggested by Syrjanen in 1983. Earlier, between 1974 to 1977, it was suggested by Zur Hansen that the virus causing con-

dylomata acuminata could be responsible for cervical cancer too[11]. The 8-kb double-stranded, circular DNA HPV genome codes two viral structural late proteins L1 and L2, and six of the early proteins: E1, E2, E4, E5, E6 and E7.The early viral proteins are regulators of the viral life cycle, as they control viral DNA replication (E1 and E2), viral transcription (E2), cytoskeletal reorganisation (E4) and cellular transformation (E5, E6 and E7).The HPV genome is comprised of three major regions: the long control region, the early (E1 to E8) genes and the late (L1 to L2) genes. Transformation of oral epithelial cells can be brought about by high risk oncogenic subtypes HPV-16, HPV-18, HPV-31, HPV-33 and HPV-35[12].

## Risk factors

Patients with HPV-positive head and neck squamous cell carcinoma (HNSCC) tend to be younger and have a lower intake of tobacco and alcohol[12]. Currently smoking and HIV-infection are both associated with significantly increased oral HPV prevalence, suggesting that tobacco-related and HIV-related immunosupression may impact oral HPV natural history (i.e. make infections more likely to persist)[13]. Patients diagnosed with this type of oral cancer were more likely to be younger (aged 40 to 55 years old), male and white[14]. Smith et al reported that the patients who are infected with HPV in the oral cavity will have a higher risk of developing cancer (Odd's Ratio = 3.7) than those who are exposed to tobacco or alcohol (Odd's Ratio = 2.6). They suggest that HPV-positive individuals who are non-smokers and drinkers are potentially at a high risk of developing cancer[15]. Sexual behaviour has consistently been associated with increased oral HPV prevalence, supporting the sexual transmission of the virus. There is evidence that HPV-positive HNSCC is a sexually transmitted disease. According to the current literature, the risk factors of HNSCC are surprisingly similar to those of cervical cancer, including the number of sexual partners, a younger age during first encounter of sexual intercourse, practice of oral sex, history of genital warts and being of a younger age[16]. Open-mouth kissing (French kissing) was associated with oral HPV prevalence in a small study of college men. The risk of developing HPV-related oral cancer may also be increased by marijuana use[16]. One family study found that a persistent oral HPV infection in mothers was associated with increased risk of persistent oral HPV infection in their infants which suggests that non-sexual transmission might occur[13].

## HPV infection

HPVs are exclusively epitheliotropic, and their replication is intimately linked to the differentiation process of the host cells[17]. After HPV inoculation, three mechanisms of infection can manifest: i) plasmid replication, which occurs in the cells of lower epithelium and may in turn subdivide into two phases; a) amplification of viral DNA up to 50 to 400 couples/diploid genome, and b) maintenance of a consistent number of couples for several cell generations. ii) Vegetative replication, which occurs in cells that differentiate from the epithelium and involves a link between cell differentiation and viral expression of the gene. iii) Productive replication, in which the virus is expelled from the epithelial cells when they undergo desquamation and is transmitted by direct contact (especially genital warts) or by indirect contact[18]. For the activation of infection, the virus must have access to the generative compartment of the epithelium through the exposure of the superficial layer, where the specific $\alpha6$ integrin receptor is present[18]. HPV infection of these cells leads to the activation of a cascade of viral gene expression resulting in the production of approximately 20 to 100 extra chromosomal copies of viral DNA per cell. This average copy number is stably maintained in undifferentiated basal cells throughout the course of the infection. Amongst the first viral proteins to be expressed are the replication factors, E1 and E2[19].

## Mechanism of HPV-induced carcinogenesis

The commonest outcome of HPV infection in humans is latent infection, which can exist for years. In this state, viral DNA is present without any clinical or histological evidence of disease. The second possible outcome of HPV infection is the formation of a benign papilloma or wart. These lesions consist of tissue hyperplasia with thickening of the spinous layer and increased capillary proliferation. Following the HPV infection of the host tissue, integration of the HPV genome occurs in the host genome and two products are formed – 'E6 protein' and 'E7 protein'[20]. The virally encoded E6 binds to a cellular ubiquitin/protein ligase, E6–AP and simultaneously to the tumour suppressor protein p53, resulting in ubiquitination of p53 and its subsequent proteolytic degradation[20]. P53 is a well-known transcription factor that induces cell cycle arrest or apoptosis in response to cellular stress or DNA damage, and it has been attributed the roles of "guardian of the genome" and "policeman of the oncogenes". While in most cancers p53 malfunction is determined by p53 mutations, in HPV-associated carcinomas wild-type functional p53 is degraded by E6 oncoproteins[21]. The E6 protein of the high-risk HPV types also plays a role in increasing cell proliferation independently of E7, through its C-terminal PDZ Ligand domain. The name PDZ is derived from the first three proteins in which these domains were found: PSD-95 (a 95 kDa protein involved in signalling), Dl g (the large protein of the Drosophila discs) and ZO1 (the zonula occludens 1 protein which is involved in maintaining epithelial cell polarity). E6 PDZ binding can mediate suprabasal cell proliferation, and this is thought to occur by uncoupling the cell proliferation and polarity control that exists in a differentiated epithelium[17]. E7 binds and destabilises the tumour suppressor retinoblastoma protein (pRb), preventing it from binding to the E2F transcription factor and it promotes the cell to enter S-phase, leading to cell-cycle disruption, proliferation,

cell cycle progression and malignant transformation. This functional inactivation of pRb results in a reciprocal overexpression of p16 tumour suppressor protein p16INK4A[20,21]. By immunohistochemistry (IHC), most HPV-positive HNSCCs show p16INK4A overexpression. In non-HPV-related HNSCC, continuous tobacco and alcohol exposure can lead to mutational loss of the p16INK4A and p53 genes. The spatial and temporal differences between high-risk and low-risk HPVs with respect to their sites of DNA replication within the epithelium are also likely to be critical. Low-risk HPVs tend to initiate DNA replication in the less differentiated cell population where elements of the cellular DNA replication machinery are still present. In contrast, high-risk HPVs replicate in the higher levels of the epithelium, and therefore require more vigorous priming of the cell division machinery[22]. Carcinogenic mechanisms in HPV-associated OSCCs may be similar to those in cervical cancers. However, since the oral cavity and the oropharynx are exposed to higher levels of chemical carcinogens compared to the genital tract, it is likely that different mechanisms are implicated in cervical and oropharyngeal carcinogenesis.

**Prevalence of HPV in OSCC**

The infection of HPV-16 is reported in 27% of oral cancer from north India and 25% to 31% from the western part of the country[23]. The prevalence of HPV in hyperkeratosis and premalignant conditions has been found to be higher than that in malignant disease, which may suggest that HPVs are more likely to act as an initiator of epithelial proliferation or play a role in the early stage of oral carcinogenesis. The detection rate of HPV 16 DNA in epithelial dysplasia (31/51; 61%) was found to be higher than that in normal mucosa (16/44; 36%) and in OSCC (30/86; 35%)[24]. The reports of HPV prevalence in oral cancer from southern India seems to be highly variable. The overall frequency of HPV infection has been reported to be 74% while 41% showed multiple HPV infection[25]. It has been proposed that an important pre-requisite for malignant transformation of these atypical squamous cells (koilocytes) is a continuous, persistent infection with HPV. This has been supported by the studies of Balram et al, who suggested that the high prevalence of HPV in OSCC point to the viral infection as being an aetiologic factor with betel quid and tobacco causing additional mutagenic steps in the carcinogenic process[25]. Microscopic evidence of koilocytes and HPV DNA (by molecular methods) have been demonstrated in around 75% of tongue cancer patients, suggesting a direct aetiologic role for HPV in these cases, in the absence of any other known factor[21].

**Diagnostic methods**

*Histopathology*

Koilocytes are histological markers for HPV. The histological diagnosis is based on HPV-related histopathological aspects, such as koilocytes, dyskeratosis, papillomatosis, hyperkeratosis, acanthosis and parakeratosis. Koilocytosis is the most common cytopathic effect and considered as a major histopathological aspect for the determination of HPV infections[26]. The presence of koilocytes in histological sections is thought to represent a cytopathic effect of HPV and is considered to give a clue to the diagnosis of HPV infection. Koilocytosis consists of the presence of abnormal koilocytes that are vacuolated with nuclear pyknosis and large clear perinuclear halos that usually occupy a greater volume of the cytoplasm. It is considered as a pathognomonic sign of HPV-associated lesions. A recent study by Cabibi et al shows that the sensitivity of detection of HPV infection by identification of koilocytes was 74% and the specificity was 72%[27].

*HPV DNA detection methods*

In-house polymerase chain reaction (PCR) methods were the first that have been designed with degenerate or consensus primers like MY09/11, GP 5/6, GP5q/6q and SPF10 targeting the highly conserved HPV DNA sequences in the L1 gene or CP I/IIG (64,111) in the E1 gene and pU in E6/7 genes. Their goal was to detect, as a whole, the majority of HPV types known, up to that period under less stringent amplification conditions. In the meantime, the Hybrid capture system and especially its latest version HC II, has become the most widespread method in labs and in clinical studies worldwide since it is simple, less technically demanding, less prone to contamination but also because it is FDA-approved. It is a non-PCR isothermal method that uses RNA probes for hybridization to denatured HPV DNA and is then followed by detection of RNA/DNA hybrids with antibodies coupled with an enzyme reacting to a chemiluminescent substrate. The method has been used in numerous method comparisons (as the only FDA approved until 2009) and performs rather well in detecting, as a whole, 13 HR types with its HR probe cocktail, despite the fact that it cross-reacts also with some LR types and given that it does not control DNA quantity and quality.

## HPV E6/7 mRNA and protein detection methods

Molecular methods detecting HPV E6/7 mRNA and/or human p16INK4A protein are targeting more advanced infections. The most widely used commercial method is Nuclisens HPV, which, by using isothermal NASBA, detects the E6/7 transcript of the five most dangerous types in three separate tubes and also checks for RNA quality. Another recently launched kit uses isothermal transcription mediated amplification technique (APTI-MA) to detect, in aggregate, the E6/7 transcript in 14 HR HPV types. Host p16INK4A protein is a surrogate marker of HPV E7 expression. The available monoclonal antibody is used in three different formats depending on the testing matrix: tissue slides, cells on a cytospin or cell extracts. P16INK4A is a cyclin-dependent kinase (CDK) inhibitor, encoded by the CDKN2A locus, which arrests the cell cycle in the G1 stage. The biologically active HPV infection in HNSCC can be determined by detecting elevated levels of p16INK4A protein by IHC. pRb inactivation by HPV E7 is associated with upregulation of CDKN2A and consequent protein overexpression. Such inactivation of pRb is very uncommon in oral cancer unrelated to HPV[21]. Therefore, p16INK4A immunostaining in conjunction with HPV DNA detection is a very useful tool to establish a diagnosis of HPV-related OSCC[28].

## HPV novel biomarkers

Novel biomarkers are urgently needed to pinpoint the low- or high-grade lesions that will progress beyond any doubt. These biomarkers should be detected early enough so that appropriate treatment in a timely manner can prevent cancer. Epigenetics is another hot area of research with groups detecting promoter CpG island hypermethylation either in L1 or E2 HPV genes or in human host genes like DAPK, TIMP3, ER, PTEN,RASSF1A, FHIT etc by various techniques, such as methylation-specific PCR and pyrosequencing of bisulfate treated DNA etc. Finally, looking at HPV 16/18 variants with DNA sequencing could yield interesting conclusions[29].

## Outcome and prognosis of HPV-associated oral cancer

Several studies have reported that detection of DNA of HPV is closely associated with poor differentiation of the tumour, positive lymph nodes and late-stage disease, which traditionally indicate poor prognosis[12]. Despite this, patients with HPV-positive HNSCC seem to have a significantly better response to chemotherapy and radio-therapy compared to HPV-negative HNSCC[30]. Recent studies have also shown that patients with HPV-16-positive HNSCC express wild-type TP53 and/or p16 have an improved disease-free survival (DFS) which supports the notion that the improved prognosis may in fact be attributed to HPV infection. Although the exact mechanism is not fully understood, three possible explanations could be: i) the genome of HPV-positive cancer cells is less unstable and/or ii) HPV-positive cells suffer from hypoxia and can be more easily induced by apoptosis or iii) treatment improves the local immunity favouring the eradication of HPV and the regression of the tumour[16]. The positive prognosis is more pronounced in HPV-positive patients who are p16-positive than in patients who are p16-negative[16]. Patients with HPV-positive oropharyngeal squamous cell carcinoma (OPSCC) have significantly better survival, although the biological reason for this remains unclear. A plausible explanation for the differences in survival between HPV-positive and HPV-negative patients is that virally driven tumours provoke an adaptive immune response directed against tumour-expressed viral antigens: immune responses against the foreign viral antigen are less likely to be suppressed; there is no central immunological tolerance to confound the immune system's attempts to control cancer[31]. HPV-16-specific CD8-positive T cells have been detected in the blood of HPV-positive OPSCC patients and, more recently, isolated from tumours, implicating a role in the anti-tumour response. Furthermore, infiltration of HPV-positive HNSCC by PD-1-expressing T lymphocytes has been shown to be a favourable prognostic factor. Cytotoxic CD8+ T cells are the principal anti-tumour effector cells, and their abundance has been shown to be a predictor of positive outcomes in several tumour types, particularly colorectal cancer, suggesting that the adaptive immune system has a role in suppressing tumour progression. The study conducted by M J Ward et al to examine the effects of HPV status and TIL levels on survival in OPSCC patients found that HPV-positive tumours were associated with significantly improved survival (3-year survival: 82% were HPV-positive vs 56%, which were HPV-negative; $P < 0.001$)[31]. The HPV-positive light-smokers or non-smokers have the best outcome in OPSCC. They also found that HPV-positive tumours in current heavy-smokers had reduced survival benefit compared with non-, ex-, or light-smokers (3-year survival: 63% HPV positive/ current heavy smokers vs 94% HPV-positive/ non-smokers, ex-smokers, and current light-smokers. However, in the study done by MJ Ward et al, there was no significant association between heavy smoking and low TIL levels.

## HPV Vaccine

The significance of vaccination as a part of prophylaxis for cervical cancer in women is well known. However, there were no data on how to prevent the transmission and expression of oncogenic HPV of the oral cavity, although there are reasons to believe that vaccinations in this field might be potentially effective. In the US, two HPV vaccines are currently available. The quadrivalent vaccine, Gardasil (HPV-4), protects against infection with HPV-6, HPV-11, HPV-16 and HPV-18. This vaccine was first licensed in 2006 for use in females aged between 9 to 26 years old for the prevention of cervical, vaginal and vulvar cancers (US Food and Drug Administration, 2011a). In 2009, licensure was expanded to also include males in this age range as clinical trial data demonstrated the vaccine's effectiveness in preventing genital warts in both genders (Centers for Disease Control and Prevention (CDC), 2010b)[27]. In addition to cervical cancer prevention, the quadrivalent vaccine is recommended, in order to reduce genital condylomas. It is thought that these HPV vaccines could have broader implications, also for other HPV-related cancer in both women and men, thereby preventing oral as well as genital infections. This has prompted many researchers to advocate vaccinating boys as well as girls, with the bivalent vaccine, to prevent HNC and by the tetravalent vaccine, to additionally prevent oral condilomatosis.

## Beyond the vaccine

HPV vaccines should eventually reduce the impact of these viruses on human health. However, vaccines may not be useful for the treatment of existing disease, and it is necessary to develop effective therapies targeting those individuals who are already infected or are currently excluded from the first phase of a prophylactic vaccination program. As there is a strong relationship between the expression of HPV E6 and E7 and cervical cancer carcinogenesis, many approaches have been directed against these oncogenes, for example, gene therapy for HPV-positive cervical cancers[32]. The development of prophylactic vaccines utilising virus-like particles to initiate immune responses offers great promise for reducing the prevalence of HPV-mediated disease in the long term[17].

## Conclusion

HPV-positive OSCC appears to be different from HPV-negative OSCC, both in its molecular and clinical features. The presence of koilocytes in the epithelium may be used as a marker for the detection of HPV in OSCC, which may be further confirmed by techniques like PCR or in situ hybridization. Also, novel biomarkers can be used for determining progression of disease. The prospect of HPV vaccine development offers hope for prevention of cancers of the oropharynx and oral cavity. HPV infection has been associated with more favourable disease outcomes, although the reason for this is not clear. Further studies are needed to dissect the HPV-positive OSCC in more detail.

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