The Association of Human Papillomavirus with Oral Lesions

By

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A thesis submitted in conformity with the requirements for the degree of Masters of Science
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Graduate Department of Dentistry
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Abstract

The oral cavity is a common site for human papillomavirus (HPV) infection, however much is still unknown about the spectrum of oral lesions associated with HPV. The prevalence of HPV was investigated in oral dysplasias and papillary lesions using a combination of ISH, p16 and MIB-1 immunohistochemistry. Results show that a subset of high grade oral epithelial dysplasias demonstrated evidence of high-risk HPV infection. Follow-up information suggests a difference in the behaviour of HPV-positive and HPV-negative high grade dysplasias. Oral papillary lesions are a heterogeneous group with variable clinical behaviour. Results support an association of low-risk HPV with papillary lesions that run a benign clinical course, despite the presence of atypia. Other oral papillary lesions with atypical features represent potentially malignant lesions that may progress to carcinoma. Potentially malignant and malignant papillary lesions are not associated with HPV infection. Infection with low versus high-risk HPV results in distinct clinical manifestations.
Acknowledgments

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<th>Description</th>
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<tbody>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>BSCC</td>
<td>Basaloid squamous cell carcinoma</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin dependent kinase</td>
</tr>
<tr>
<td>CIN</td>
<td>Cervical intraepithelial neoplasia</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>E</td>
<td>Early region</td>
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<tr>
<td>FEH</td>
<td>Focal epithelial hyperplasia</td>
</tr>
<tr>
<td>FFPE</td>
<td>Formalin fixed paraffin embedded</td>
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<tr>
<td>G₀</td>
<td>Cell cycle latent phase</td>
</tr>
<tr>
<td>G₁</td>
<td>Cell cycle first gap</td>
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<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HNSCC</td>
<td>Head and neck squamous cell carcinoma</td>
</tr>
<tr>
<td>HPV</td>
<td>Human papillomavirus</td>
</tr>
<tr>
<td>HR HPV</td>
<td>High risk human papillomavirus infection</td>
</tr>
<tr>
<td>HSIL</td>
<td>High grade squamous intraepithelial lesion</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>INF</td>
<td>Interferon</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ISH</td>
<td>in situ hybridization</td>
</tr>
<tr>
<td>L</td>
<td>Late region</td>
</tr>
<tr>
<td>LC</td>
<td>Langerhans cell</td>
</tr>
<tr>
<td>LCR</td>
<td>Long control region</td>
</tr>
<tr>
<td>LR HPV</td>
<td>Low risk human papillomavirus infection</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>OSCC</td>
<td>Oral squamous cell carcinoma</td>
</tr>
<tr>
<td>ORFs</td>
<td>Open reading frames</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>pRb</td>
<td>retinoblastoma protein</td>
</tr>
<tr>
<td>PSCC</td>
<td>Papillary squamous cell carcinoma</td>
</tr>
<tr>
<td>PVL</td>
<td>Proliferative verrucous leukoplakia</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase PCR</td>
</tr>
<tr>
<td>S</td>
<td>Synthetic phase of cell cycle (DNA synthesis)</td>
</tr>
<tr>
<td>SCC</td>
<td>Squamous cell carcinoma</td>
</tr>
<tr>
<td>UADT</td>
<td>Upper aerodigestive tract</td>
</tr>
<tr>
<td>VC</td>
<td>Verrucous carcinoma</td>
</tr>
<tr>
<td>VLPs</td>
<td>Virus-like particles</td>
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Chapter 1.  Introduction

1.1 Biology of Human Papillomavirus

Human papillomaviruses (HPV) are epitheliotropic, double stranded DNA viruses known to infect and cause lesions in a variety of anatomic sites, including the skin, anogenital, and upper aerodigestive tract mucosa. More than 100 different subtypes of HPV have been identified, and these are stratified into high and low risk groups depending on their relative ability to induce carcinogenesis (1). Specific HPV subtypes from both high and low risk groups are associated with epithelial lesions found on the skin and mucosa. Low risk HPV types (2, 4, 6, 11, 13, 32) are associated with benign verrucal-papillary lesions, while high risk HPV types (16, 18, 31, 33, 35, 58) have been demonstrated in a number of potentially malignant and malignant lesions, including: cervical intraepithelial neoplasia, cervical squamous cell carcinoma, and a subset of oropharyngeal carcinomas (2-7).

1.1.1 The HPV Genome

The HPV viral genome is non-enveloped, circular, and has an approximate size of 8Kb (8,9). The viral genome is transcribed from a single strand of viral DNA, which consists of 8 open reading frames (ORFs) (9). The genome contains 3 main parts; the early region (E), late region (L) and a non-coding long control region (LCR) (9). The E region encodes viral proteins E1-E7, which control the transcription of viral DNA, while the L region encodes viral proteins L1 and L2, which control assembly of the virion and viral spread (9,10). The replication factors E1 and E2 are amongst the first viral proteins that are produced (8), and are thought to be responsible for regulating the replication and transcription of viral DNA (11). It is thought that E4 and E5 are involved in the regulation of late viral functions, however their exact role remains unknown (8,12). E6 and E7 target the host proteins pRb and p53, which regulate progression through the cell cycle (refer to section 1.1.3 for more detail) (13,14). The interaction of E6 and E7 with their molecular targets thus preserves the replicative potential of infected cells (8,14).
1.1.2 The HPV Life Cycle

HPVs demonstrate selective affinity for keratinocytes, infecting the basal and parabasal cells of squamous epithelium (15). It is most likely that the virus gains access to basal cells via microscopic breaks in the mucosa (8). The life cycle of HPV is closely associated with the differentiation of epithelial cells, and production of mature viral particles only occurs in terminally differentiated keratinocytes (8). It is speculated that the microenvironment associated with the maturation of keratinocytes from the basal layer to the surface promotes viral replication, allowing keratinocytes to act as permissive cells (15). Specifically, HPV infection leads to activation of viral gene expression, resulting in production of extrachromosomal copies of viral DNA, or episomes, which are maintained at low copy numbers in the undifferentiated basal cells as long as the infection persists (8). When the cells of the basal layer divide, one daughter cell remains in the basal layer, while the other moves up and begins to terminally differentiate (16). Viral DNA segregates between the two daughter cells during cell division, and replicates to maintain copy numbers (16). In order for the virus to continue replicating in a differentiated cell which contains little to no replicative machinery, the virus expresses proteins early in infection in the basal epithelial compartment, which stimulates G1 to S phase progression (8). In addition to maintaining replicative potential, the virus also requires cells to be partially differentiated, as the late promoter, responsible for transcribing the mRNA coding for capsid proteins, is mediated in differentiated cells (8,16). In order to overcome this problem, the virus uncouples the G1 to S phase progression from differentiation, and both processes occur in the same epithelial cell (16). The assembly of viral particles takes place in the superficial epithelial layers following late gene transcription and translation, and the infected cells are desquamated from the top of the epithelium (16).

1.1.3 Oncogenic Activity of HPV

The pathogenesis of HPV related cancers is related primarily to the transforming activities of the E6 and E7 oncoproteins in high risk HPV types (14,17). Although E6 and E7 are also present in low risk HPV, less is known about the function of these proteins in the context of latent and subclinical infections (8,18). E7 binds to and degrades pRb, which prevents inhibition of the E2F transcription factor, leading to loss of cell cycle control (7). E6 targets the tumour suppressor gene p53 and inhibits it, therefore preventing p53-dependent apoptosis and cell cycle arrest (14).
The precise way in which high risk E6 and E7 proteins differ from low risk E6 and E7 proteins, and how this relates to their transforming capabilities, is not entirely known (18). It has been suggested that high risk E6 and E7 have greater affinity for cell cycle regulators, and thus produce a more profound deregulation of the cell cycle (18). Loss of function of p53 and pRb are two of the most commonly identified abnormalities in the development of human cancers, and it is the direct effect of the HPV viral proteins on these pathways that leads to the development of HPV related cancers (14,19). Loss of cell cycle regulatory mechanisms in persistently replicating HPV infected cells allows for the progressive accumulation of genetic damage and mutations, which subsequently lead to malignant change (14,20).

1.1.4 Physical State of the Viral Genome

When exposure to HPV results in infection, clinically normal looking mucosa may contain a low number of viral episomes within the basal epithelial compartment (18,21). In general, HPV associated benign lesions demonstrate low risk HPV DNA in episomal form, while high risk HPV DNA in malignant lesions is often, though not exclusively, integrated within the cellular genome (16,22). Studies from the uterine cervix have demonstrated that up to half of high risk HPV related cancers have integrated HPV (14). Viral genome integration is thought to disrupt the repressive function of E2, leading to increased expression of E6 and E7 (14,18,23-25). The increased expression of E6 and E7 leads to increased cellular proliferation, and thus, integration of the viral genome is considered a critical step in the progression to cancer (14,19). It is also thought that integration may contribute to the stability of E6 and E7 mRNAs, and therefore cells containing integrated genomes may have a growth advantage over those with episomal forms (14,26,27). Interestingly, significant portions of the viral genome, including E5 may be deleted upon integration, suggesting that they do not play a role in malignant transformation (28).

Investigations have also revealed that the coexistence of both episomal and integrated forms may be important in HPV mediated carcinogenesis (14,29). Mixed forms of HPV 16 DNA, both integrated and episomal, have been reported in both cervical and head and neck squamous cell carcinoma (HNSCC) (30). Recent studies have suggested that the expression of E1 and E2 viral proteins in episomal HPV DNA may induce and amplify chromosomal abnormalities by commencing DNA replication from the integrated viral genome (14,29).
1.1.5 Immunologic Response to HPV

Despite its ubiquity, progression from clinically detectable infection to invasive cervical carcinoma occurs in a minority of cases, currently thought to be around 1% (31). The precise circumstances that determine which infections transform to cancer and which infections are cleared by the host are complex and most likely multifactorial (31). Current evidence suggests that a deficient immune response to HPV infection may be important for viral persistence, an important and well established risk factor for the development of cancer (28,31,32). Much of what is known about the role of immunity in the progression of HPV induced lesions has been indirectly supported by investigations in immunosuppressed patients. For example, individuals with HIV infection demonstrate increased overall persistence and prevalence of HPV infections, decreased clearance, and infection with HPV types not common in immunocompetent persons (28,33).

1.1.5.1 Mechanisms of Immune Evasion

HPV related immune evasion appears to involve multiple aspects of both innate and humoral immunity. Avoiding antigen presentation is one of the main ways that HPV avoids detection by the immune system (34). Replication of the virus occurs without cytolysis or necrosis of the host keratinocyte, which prevents presentation of viral antigens (31,35). HPV exclusively infects epithelium, and has no viremic or blood-borne phase, further limiting the ability of the immune system to detect the virus and mount an appropriate response (31,36).

Theoretically, antigen presenting cells (APCs) native to the epithelium, or Langerhans cells (LC) should detect the virus, however, there is evidence to suggest that LC are not activated by the uptake of HPV capsids (35). Recombinant HPV-like particles are produced by the overexpression of the HPV capsid protein L1(37), and it has been shown that LCs do not demonstrate an epitope-specific immune response against L1 antigens when incubated with L1 virus-like particles (35,38). Contrary to what might be expected, HPV capsids do not appear to elicit a significant immune response, as it is thought that HPVs have evolved methods to limit expression of capsid proteins to superficial differentiated epithelial cells, thus avoiding detection by LCs (31,34).
HPVs are also known to disturb specific functions of the immune system, particularly the function of interferons. The activation of type 1 interferons (INF) is an important part of the innate immune response, and is normally triggered by viral infection (31,35). E6 and E7 have been shown to prevent immunoregulation by INF-α and INF-β, which normally function to activate immature dendritic cells (DC), triggering the adaptive immune response (31,35,39). Furthermore, infection with high risk genotypes has also been shown to down regulate INF-α inducible gene expression (35,40).

The HPV proteins themselves have also been associated with suppression of the immune response (34). For example, studies have shown that immunocompetent women with cervical cancer have a poor cell-mediated response to E7, which strongly suggests that E7 expression may cause evasion or even suppression of the normal immune response (31). It is speculated that this may occur through a number of mechanisms, including: limited transcription before DNA integration, minimal levels of oncoprotein expression, and sequestration of E7 in the nucleus where it cannot be accessed by antigen presenting cells (APCs) (31).

Peripheral tolerance is another mechanism theorized to contribute to the immune evasion behaviour of HPVs. Normally, pro-inflammatory cytokines stimulate the maturation of DCs and subsequent transmission of a stimulatory or immunogenic signal to T cells (31). In the setting of high risk (HR) HPV infection, however, a relative lack of inflammation primes immature DCs to transmit a tolerance favoring, or tolerogenic, signal to T cells carrying E7 receptors in draining lymph nodes (31). In this way, the virus is able to escape the T cell regulatory network that would normally elicit an adaptive immune response (31). Molecular mimicry may contribute to low immunogenicity of E7, which shares antigenic motifs with several human proteins (31,41)

In summary, all of the mechanisms discussed above have the overarching purpose of limiting opportunities for the detection and presentation of viral antigens in order to mount a sufficient immune response (31).

1.1.5.2 Immune Response to HPV and Vaccine Development

Paradoxically, despite well evolved mechanisms for immune evasion, most HPV infections resolve spontaneously with time as a result of a successful antigen-specific, cell mediated
immune response (34). In animals, seroconversion and production of antibodies against the L1 capsid protein follows the cell mediated immune response (36,42). While it is theorized that this also occurs in humans after natural infection, antibody concentrations are low, and many women never show seroconversion (35). Difficulty with assay standardization and sensitivity make it difficult to measure the true rate of seroconversion, however it is estimated that 30-50% of women with evident persistent genital HPV infections never seroconvert (34,35,43-46). Despite the fact that natural antibody responses to HPV infection are poor, neutralizing antibodies against the major viral capsid protein L1 have proven protective when L1 is given as a prophylactic vaccine in individuals without established HPV infection (35,47). This type of vaccine strategy is predicated on the self-assembly of recombinant L1 proteins into virus-like particles (VLPs) that do not contain genetic material (33,48). Nearly all individuals that are vaccinated show seroconversion, and the antibody concentrations elicited by the vaccine appear to be significantly greater than those achieved by natural exposure (35). Mucosal protection is most likely a result of antibody presence in the mucosal secretions, however, the precise manner by which antibodies prevent viral entry into the mucosa is unknown (33,35,49). Prophylactic vaccinations have shown efficacy for up to 5 years, however the extent of long term protection is not definitively established (35,50). At present, immunization with capsid proteins fails to demonstrate a therapeutic effect for established infections, and it is thought that an immunization strategy that centres on cell mediated immunity, specifically antigen-specific T cell mediated immunity is likely more appropriate in the treatment of established HPV infection (37). Several therapeutic HPV vaccines that target the oncogenic proteins E6 and E7 are currently under development, with animal studies demonstrating enhanced HPV specific immunity and anti-tumour responses (37). Clinical trials with several candidate vaccines are also underway (37).

1.1.5.3 Vaccination for the prevention of HPV Related Head and Neck Cancers

Unlike in the uterine cervix, the natural history of oral HPV infections is not well characterized (51). Persistence of viral infection is established as a major risk factor for malignant progression in the cervix, and while it is likely that this is also true of oral infections, much is still unknown about the clearance rates of oral infections, and how differences in the local tissue environment
(e.g. presence of lymphoid tissue in Waldeyer’s ring) may affect infection (37,51). Natural history studies are essential to further understanding of the potential interrelationship of age, sex and modifiable risk factors, such as smoking and sexual history on oral infections (52). Furthermore, there is some data that suggests that the natural history of oral HPV infections may be dissimilar to cervical HPV infections (51,53). For example, D’Souza and colleagues (53) found that tobacco use, current anti-viral treatment in HIV infected patients, and increasing age were associated with increased risk of persistent oral, but not cervical HPV infection at follow-up. Therefore, at present, as vaccine efficacy against oral HPV infection is not known, it is not recommended for the prevention of HPV positive HNSCC (52).

1.2 Epidemiology

1.2.1 Risk factors for HPV Infection and Transmission

Gillison and colleagues have published detailed reports on the epidemiology of oral HPV infections (52). Transmission of HPV is thought to occur predominantly by sexual contact. In their large, cross-sectional study of a US population, Gillison et al. found that HPV infection is more prevalent in sexually experienced versus inexperienced individuals, and increases significantly with the number of sexual partners. However, they also found that oral HPV infections are present in sexually active individuals who do not engage in oral sex, and rarely even in sexually inactive individuals, suggesting that alternate means of transmission may occur. If transmission does occur by casual contact, it is thought to be uncommon. The International Head and Neck Epidemiology consortium (INHANCE) study on sexual behaviour and the risk of head and neck cancers found associations between certain sexual behaviours and increased odds of oropharyngeal cancer, but not oral cavity, laryngeal or hypopharyngeal cancers (54).

According to Gillison et al. (52) the main risk factors for oral HPV infection of any type included: age (bimodal, 30-34yrs and 60-64 years), male sex, lifetime number of sexual partners, and smoking. Marijuana and heavy use of alcohol were only reported as significant risk factors by univariate, but not multivariate analysis. With respect to smoking, it was found that individuals who are current and more frequent smokers are more likely to have oral HPV infection. Further, studies of HPV infection in the cervix have shown that smoking increases the
risk of developing high grade squamous intraepithelial lesions (HSIL) and cervical carcinoma, and the risk of developing cervical carcinoma is associated with increasing overall exposure to smoking (55).

1.2.2 Prevalence of Oral HPV Infection in the General Population

In general, detection of HPV requires epithelial samples that can be obtained by tissue biopsy or exfoliated epithelial cells. Oral HPV detection is accomplished by the examination of exfoliated cells collected by direct swabbing or in oral rinse or saliva samples (56). Because of the ability to collect exfoliated cells over a larger area, oral rinsing is considered to be the most sensitive method for oral HPV detection, however it is unknown how behavioural factors such as recent food consumption and oral hygiene may influence the detection of HPV (56,57). Historically, estimates of HPV prevalence in normal individuals have been variable, presumably because of heterogeneity in HPV detection methods (refer to section 1.3). Preliminary investigations in this area produced conflicting results, with prevalence rates ranging from 0-70% (11,58-64). Larger population based studies, however, estimate that the overall prevalence of HPV in the oral mucosa in normal individuals is approximately 5-10%, which is substantially lower than in the cervix (11,52,56,65-70). Identification of HPV in the oral mucosa of otherwise healthy individuals suggests that, as in the cervix, not all oral HPV infections progress to malignancy (11). Recently, Gillison et al. (52) conducted a cross-sectional study of the prevalence of oral HPV infection in a US population, and found that overall prevalence for men and women aged 14-69 years was approximately 6.9%, however, prevalence of HPV type 16 infection was only 1.0%. As the natural history of oral infections is not well understood, the risk of oral or oropharyngeal carcinoma in individuals who have demonstrated high risk HPV infection in their saliva has not been established definitively (refer to section 1.5).

1.3 HPV Detection Methods

The current approach to examine cell and tissue samples for HPV is based on detection of viral DNA or RNA. Immunohistochemical staining for HPV capsid antigen, or for E6 and E7 proteins is less sensitive and not commonly used at this time (9). Southern and northern blots were originally considered the techniques of choice for HPV DNA and RNA detection, but several limitations, including: long assay time, lack of universal applicability to formalin-fixed paraffin
embedded (FFPE) specimens and requirements for large amounts of tissue, have made these techniques obsolete (71). Current detection methods are broadly classified as either target amplification or signal amplification methods (72). Target based methods involve the amplification of a nucleic acid sequence by the use of nucleic acid polymerases, primers and nucleotides (71). Polymerase chain reaction (PCR) is the most commonly used target amplification method for HPV detection. Signal amplification methods involve the hybridization of viral nucleic acids with target specific probes that are visualized in situ, as with chromogenic in situ hybridization (ISH) assays (71).

1.3.1 Consensus PCR and Real-Time PCR

Broad spectrum or consensus type PCR allows for the detection of multiple different HPV types in one assay, which is accomplished by the selection of primers directed against the L1 or E1 open reading frames, which are highly conserved sequences of the HPV genome (71). The detection of viral DNA by non-quantitative PCR, while specific, does not allow distinction between transcriptionally active and inactive infections, which limits clinical relevance (73). As the carcinogenic potential of HR HPV is thought to be directly related to the expression of viral oncogenes E6 and E7, the presence of HPV DNA in and of itself does not exclude possibility of a transient infection with no transformation potential (74). Furthermore, though PCR based assays are highly sensitive and often yield a high prevalence rate for HPV infection, the likelihood of false positivity has been recognized (75). The clinical utility of targeting the L1 gene with PCR based methods has also been questioned, as multiple portions of the HPV genome, including L1 may be lost upon viral integration into the host genome, which would lead to false negative results (76,77).

Real time PCR amplifies HPV sequences using wide spectrum or type specific primers, and assay parameters are adjusted to give a quantitative relationship between the amount of target and the amount of amplified product (77). Real time PCR allows for greater control over sample contamination, a noted problem with PCR based methods, by eliminating the need for post-PCR manipulation (77,78). Further, this technique allows for quantification of HPV viral load, which may be clinically important, as higher viral loads are thought to be necessary for establishing a clonal relationship, while low viral loads may more in keeping with a latent, non-transforming infection (75,77).
1.3.2 Viral mRNA Detection

As persistence of E6 and E7 expression is thought to be a necessary requirement for malignant progression, detection of E6 and E7 mRNA in frozen tissue is generally regarded as the gold standard for identifying transcriptionally active HPV infection (71,73,74). The potential clinical utility and importance of this type of assay was recently confirmed by Lingen and colleagues (79) who demonstrated that only half of their cases of oral SCC that were positive for HPV DNA by PCR also showed evidence of viral E6/E7 expression. Despite its accuracy, viral mRNA detection may not be universally accepted for clinical use, as it is more technically challenging in FFPE specimens than other methods, and requires significantly more tissue (56,80,81).

1.3.3 In situ Hybridization

The main advantage of in situ hybridization (ISH) methods for HPV detection over other HPV detection methods relates to the ability to visualize HPV DNA directly within the nuclei of lesional tissue (77). The presence of a punctate hybridization signal is indicative of integration of the viral genome, while a homogeneous hybridization signal indicates persistence of the episomal form (56,82,83). ISH-based methods can be reliably applied to FFPE tissue, and chromogenic ISH results can be visualized using a conventional light microscope (84). Low sensitivity has been identified as a potential limitation of ISH based methods when compared to PCR based methods (83,84), and could lead to unacceptable false negative detection rates. However, the introduction of signal amplification steps has reportedly improved the sensitivity of ISH, allowing for viral detection of a copy number as low as 1 viral copy per cell (83,84).

1.3.4 Hybrid Capture

Hybrid capture is used extensively for the detection of HR HPV types in cervical cytology specimens, and involves the hybridization of chemically denatured, single stranded viral DNA to specific RNA probes, producing a visible hybridization signal (77). Given that the use of brush biopsies for oral HPV detection is not currently validated for clinical use, the use of hybrid capture is limited for the evaluation of head and neck specimens (77).
1.3.5  p16

p16 is a member of the INK4 family of cyclin dependent kinase (CDK) inhibitors (85). p16 is a negative regulator of the cell cycle, and is involved in the functioning of the Rb protein pathway (85-88). Under normal circumstances, p16 expression causes hypophosphorylation of pRb, leading to the binding of and inhibition of the E2F transcription factor, and arrest of the cell cycle at the G1/S transition (85,86). p16 has also been related to other cellular processes such as apoptosis and angiogenesis (85).

1.3.5.1  p16 Expression in Tumours

p16 staining is widely used as a surrogate marker of transcriptionally active HPV infection (74). Loss of p16 function as a result of inactivation is recognized as an important event in progression of many human cancers including oral SCC (85,89). In HPV related neoplasms of the uterine cervix and oropharynx, overexpression of p16 characterizes high risk HPV infection, and is used as an adjunct tool for diagnosis (85,90). p16 overexpression in HPV related tumours is related to viral E7 oncoprotein expression and inactivation of pRb (74). The inactivation of pRb releases p16 from normal regulatory feedback mechanisms, and subsequently p16 protein levels rise in an attempt to overcome uncontrolled cell proliferation (85). Essentially, p16 overexpression is a manifestation of a failed attempt at blocking cell proliferation (91).

1.3.5.2  p16 Immunohistochemistry for the Detection of High Risk HPV

Immunohistochemical staining for p16 is cost-effective and reliably applied to FFPE tissues. HPV detection using p16 has been shown to be highly sensitive (100%), however its specificity is only 79% (74). p16 overexpression in the absence of HPV 16 infection may represent disturbances to the pRb pathway that are unrelated to HPV, or the presence of other HPV subtypes (73). Currently, the use of p16 as a stand-alone method for the detection of HPV in HNSCC is not supported by the literature (79). Though p16 has been reported to have a positive predictive value approaching 93% for HPV positive oropharyngeal cancers, this falls to 41% for HPV positive oral cavity carcinomas (79). These results suggest that there may be important differences in site specific expression that have not yet been fully characterized.
1.3.6  **Ki-67/MIB-1: A Proliferation Marker**

The Ki-67 protein is expressed in the active phases of the cell cycle, but is absent in $G_0$ (92). Though much of the function of the Ki-67 protein is unknown, it is thought to perform a fundamental role in cell proliferation, and removal of the protein leads to loss of proliferation activities (93,94). MIB-1 is a monoclonal antibody directed at recombinant parts of the Ki-67 antigen, which demonstrates an identical staining pattern to the Ki-67 antibody, and is thus considered equivalent (95). The proportion of cells committed to the cell cycle, or growth fraction, can be assessed by Ki-67/MIB-1 staining (95-97).

1.3.6.1  **Ki-67 and HPV**

Expression of the E6 oncoprotein associated with HR HPV infection is known to cause the degradation of p53 (14). P53 is a tumour suppressor gene important in the regulation of the cell cycle, DNA repair and apoptosis (2,98). Loss of p53 activity as a result of HR HPV infection, therefore, may result in increased and unregulated cellular proliferation (2). In oral mucosa, Pillai and colleagues found that cell proliferation increased with the degree of histologic abnormality, and that increased proliferation was a feature of HPV infection (2). Similarly, in the cervix, Mimica *et al.* (99) found that HR HPV positive cervical intraepithelial neoplasia (CIN) exhibited significantly higher Ki-67 labelling of the upper and middle portions of the epithelium when compared with HPV negative and low risk (LR) HPV cases. Furthermore, they found that Ki-67 labelling was significantly different between grades of CIN. The association of increasing Ki-67 positivity with severity of dysplasia has also been confirmed in other studies of the cervical mucosa (100,101). With respect to oropharyngeal lesions, El-Mofty *et al.* (102) reported that higher Ki-67 labelling indices were a distinguishing feature of HPV positive non-keratinizing SCC as compared to HPV negative carcinomas.

1.3.7  **Diagnostic Algorithms for HPV Detection**

There are currently no standardized methods for HPV detection in HNSCC (77). Singhi and Westra advocate for the use of p16 immunohistochemistry (IHC) as a screening method for HR HPV, followed by the use of ISH (84). This type of algorithm takes advantage of the high sensitivity of p16 and uses ISH as a secondary, more specific assay for confirmation (77). Comparison of ISH based methods to other methods of HPV detection supports its use both as a
practical and clinically relevant assay. A recent study demonstrated a concordance rate of 86% between HPV 16 DNA ISH and HPV 16 E6 mRNA PCR in oropharyngeal carcinomas (80). Similarly, Jordan et al. (81) found that when combined with p16 IHC, HPV 16 ISH had a very high specificity compared with E6/E7 expression by PCR, with a false positive rate of approximately 3%. There is one caveat to this study, however, because the HPV probe used in their study is not commercially available. Schlecht et al. (103) compared ISH based methods with p16 IHC for detecting HPV in head and neck cancers. Although they found that ISH based methods were less sensitive and specific when compared with PCR detection of viral E6/E7, they did not consider PCR versus combined ISH and p16 in their comparisons.

Some investigators have utilized methods of HPV detection that combine p16 IHC, ISH and PCR to detect HPV infection in HNSCC (79,80,104). Pannone et al. (104) investigated the reliability of p16 IHC, ISH and consensus HPV DNA PCR. They found that the addition of ISH increased sensitivity for HPV detection over the use of p16 and consensus PCR alone. Lingen et al. (79) used a combination of consensus PCR and type specific PCR, reverse transcriptase PCR for E6/E7 and p16 immunohistochemistry to detect HPV in oral SCC. ISH was also used to assess the specificity of HPV for the nuclei of tumour cells. Forty cases of 409 were positive for HR HPV DNA, 27 of which remained positive when confirmed with type specific PCR for HPV subtype. When E6/E7 expression was evaluated, 24 cases were positive. ISH was then used to confirm localization of the viral DNA to tumour cell nuclei, which demonstrated positivity for 20 of the 24 cases that were positive for E6/E7. p16 was positive in 19 of the 24 HR HPV E6/E7 positive tumours, and 27 of the 385 HPV negative tumours. The results of this study support the concept that consensus PCR, while sensitive, will lead to overestimation of the prevalence of biologically relevant HPV infection. Wei et al. (80) used a combined approach of PCR for E6 mRNA, p16 and ISH. They found that ISH and E6 mRNA had a strong concordance (86%), but did not have perfect correlation. Explanations for discordance included sampling differences, and the presence of ISH signals in nearby hyperplastic epithelium, which could produce a positive result for E6 mRNA, but be scored as ISH negative (80). Thus it appears that the use of mRNA in combination with p16 and ISH may improve diagnostic accuracy, however technical barriers in FFPE tissues (refer to section 1.3.2) likely explain why this approach is not used more universally.
1.4 The Association of HPV with Lesions of the Head and Neck

1.4.1 Association of HPV with Benign Oral Lesions

Low risk (LR) HPV types are associated with several benign oral lesions, including: squamous papilloma, condyloma accuminatum, verruca vulgaris, and focal epithelial hyperplasia (2,5,18,21). Squamous papillomas occur commonly in the oral cavity. Clinically, they form a round to oval, discrete, exophytic mass with numerous finger-like excrescences (105). Histologically, they show a papillary configuration with keratinized or non-keratinized stratified squamous epithelium covering fibrovascular cores (105). Association of a viral etiology of squamous papillomas was reported in the literature as early as the late 1960’s (106,107). The overall detection rate of HPV DNA in oral papillomas, most commonly types 6 and 11, is currently estimated at close to 50% (107,108), however it appears that rates may vary depending on the type of HPV detection methods used. Eversole and colleagues found that 5% (1/20) of oral papillomas in their series showed evidence of viral antigen by IHC, and viral DNA by ISH was present in 35% (7/20), despite the fact that viral cytopathic effects (koilocytes), were evident by light microscopy in 45% of cases (109). Fregonesi et al. found evidence of LR HPV DNA by ISH in only 14% (2/14) oral squamous papillomas (110). Syrjanen et al. found that HPV DNA was present in 33.8 % (21/62) of squamous papillomas and condylomas collectively. It is likely that this is an overestimation of the true prevalence of HPV in papillomas, as studies have shown that HPV viral DNA is more common in condylomas than in squamous papillomas (109,111). Oral papillomas generally show self-limited growth, behave indolently and usually do not recur after excision. Rarely, lesions measuring up to 3 cm have been reported (105). Malignant transformation has not been described in untreated lesions (105).

Condyloma accuminatum is considered to be a manifestation of a sexually transmitted disease, and lesions are known to affect both the anogenital and oral regions (105). Oral condylomas are much less common than squamous papillomas. Condylomas also have a warty appearance, but tend to have more blunted surface projections, are larger than papillomas, and are often present in clusters (105). Histologically, condylomas show a papillary architecture consisting of connective tissue cores covered by acanthotic stratified squamous epithelium, often with koilocytosis (105). It can be very difficult to distinguish between squamous papilloma and condyloma accuminatum without a history of orogenital contact, however, more pronounced
acanthosis, parakeratin crypt formation and more extensive koilocytosis can be helpful (109). Condylomas are most commonly associated with low risk HPV subtypes including types 6, 11, 2, 53 and 54. Infection with high risk types 16, 18 and 31 has been described, but is uncommon (105,112,113). Although few large scale studies exist, Syrjanen reported that of 116 oral condylomas studied for HPV DNA in the literature, 75% demonstrated evidence of HPV involvement (107,108). Condylomas are contagious and may spread by autoinoculation to other oral sites; therefore excision is recommended (105). Although anogenital condylomas infected with HR HPV types 16 and 18 are associated with increased risk of malignant transformation, this relationship has not been definitively established in the oral mucosa (105).

Verruca vulgaris is a common cutaneous lesion found in children, but can also be found in the oral mucosa. Clinically, these lesions are often small, white, and well-demarcated. Verrucas can be solitary or multiple. They commonly appear on the vermillion border, labial mucosa or anterior tongue, presumably spreading by autoinoculation (105). Verruca vulgaris characteristically shows hyperkeratosis with hypergranulosis, and a convergence or “cupping” of acanthotic rete ridges (105). Evidence for HPV involvement in verruca vulgaris was first demonstrated using electron microscopy (114). Reported rates of HPV detection vary in the literature from 54-100%, and HPV subtypes 2 and 57 are the most commonly detected (107,108). Oral verrucas are treated by surgical excision. A small number of treated verrucas may recur, but malignant transformation is not reported (105).

Focal epithelial hyperplasia (FEH) occurs primarily in the oral mucosa of several ethnic populations, including in individuals of aboriginal heritage (105). Clinically, FEH classically presents as clusters of flat topped nodules or “cobblestones” (105). Histologically, FEH demonstrates acanthosis of the squamous epithelium with club or battle-axe shaped rete ridges (105). HPV types 13 and 32 are the most commonly detected subtypes in these lesions, and the rate of HPV detection is estimated at approximately 80% based on a survey of the literature (108). FEH usually resolves spontaneously, however this may take months to years (105). The risk of recurrence is low, and malignant transformation does not occur (105).
**1.4.2 HPV Association with Head and Neck SCC**

It is now well established that HR HPV, particularly type 16, plays a causative role in the development of a subset of head and neck squamous cell carcinomas (HNSCC). HPV positive HNSCC show distinct epidemiologic, molecular and risk factor profiles when compared to HPV negative tumours. HPV positive tumours show a striking predilection for the oropharynx, particularly the tonsils and base of tongue (115-118). It is currently estimated that approximately 70% of tumours in the oropharynx demonstrate infection with HR HPV (119).

Several investigators have reported associations between HR HPV and laryngeal cancer (118,120). A recent systemic review reported a weighted prevalence of 23.6% for laryngeal cancers, incorporating data from 41 studies (121). Similarly, HPV 16 has been reported in malignancies of the nasal cavity (122). Mork *et al.* (123) investigated the role of HPV infection on the risk of SCC of the head and neck. Using PCR based methods HPV 16 DNA was identified in one case each of laryngeal and nasal cavity SCC, or 3% and 14% of all carcinomas at these two sites, respectively. Presently, cause and effect relationships have not been definitively established at non-oropharyngeal UADT sites, though it is clear that the overall contribution of HPV to carcinogenesis is substantially less than in the oropharynx (56,123).

Both HPV negative and HPV positive HNSCC are more prevalent in males, however HPV positive tumours affect men with an average age approximately 5 years younger than conventional non-HPV related HNSCC, and the difference in age is statistically significant (124). HPV 16 positive HNSCCs have been independently associated with several measures of sexual behaviour, including: lifetime number of sexual partners, infrequent barrier use and history of infection with a sexually transmitted disease (124). HPV 16 positive HNSCC has also been associated with marijuana exposure, but not with cumulative measures of tobacco use, alcohol consumption or poor oral hygiene (124). Conversely, HPV-16 negative HNSCC has been associated with smoking, alcohol consumption and poor oral hygiene, but not sexual behaviour or marijuana use (124).

From a molecular perspective, HPV positive HNSCCs express viral E6 and E7, are less likely to have p53 mutations, and show overexpression of p16 by IHC (118,125). HPV positive and HPV
negative HNSCC also differ in prognosis. HPV positive oropharyngeal carcinomas are associated with improved disease specific survival, and Gillison et al. found a 59% lower risk of disease associated death in HPV positive versus HPV negative cancers (118). There is also evidence that HPV positive tumours may be more responsive to certain types of treatment. Fakhry et al. (126) demonstrated that individuals with HPV positive tumours had statistically significant higher response rates to induction chemotherapy and chemoradiation when compared to those with HPV negative tumours.

From a histopathological standpoint, HPV positive oropharyngeal carcinomas are most accurately described as non-keratinizing squamous cell carcinomas (127). They demonstrate several characteristic histologic features, including: basaloid morphology, lobular growth pattern, permeation by infiltrating lymphocytes, and significantly, lack of association with premalignant lesions of the overlying epithelium (82). It is important to recognize that while characteristic, the association with basaloid morphology is not exclusive to HPV positive oropharyngeal carcinomas, and other tumours, particularly basaloid squamous cell carcinomas (BSCC) may share considerable morphologic and anatomic overlap (127). BSCC is an aggressive subtype of SCC that occurs with some frequency in the oropharynx, as well as at other UADT sites. Begum and Westra (127) found BSCC of the head and neck to be a heterogenous mixture of HPV positive and negative tumours that could not be separated based on morphology alone. Their findings of significantly decreased survival in HPV negative versus HPV positive BSCC strongly support the use of HPV 16 ISH to distinguish between these two tumour subsets. They also point out the potential for confusion associated with using the term “basaloid” as a descriptor. HPV positive tumours frequently show higher mitotic activity and increased cell cycling, as demonstrated by cell cycle markers such as Ki-67(102). The identification of a distinctly non-keratinizing histological phenotype in HPV positive oropharyngeal carcinomas initially led to the belief that these tumours were poorly differentiated (82). However, it is now recognized that HPV positive tumour epithelium recapitulates the reticulated epithelium lining the tonsillar crypts rather than stratified squamous epithelium, and therefore the non-keratinizing histomorphology supports the interpretation of a highly differentiated tumour process (128).
1.4.2.1 HPV in Oral Squamous Cell Carcinoma

In contrast to the oropharynx, current evidence for the role of HPV in the development of oral squamous cell carcinoma (OSCC) is much more equivocal. The reported prevalence of HPV infection in OSCC is highly variable, ranging from 0-100% (129-132). This variability has been explained largely by differences in sample type, storage medium, HPV detection assay, and anatomical location (11,15,75,133). Recently, several studies have attempted to re-examine the prevalence of HPV in OSCC while controlling for these factors. Overall, the evidence suggests that the rate of HPV infection in oral cavity carcinomas is much lower than in the oropharynx, ranging from 2-15% (75,134-137). Machado et al.(134) found that HPV was uncommon in oral cavity carcinomas, with only 3.78% (2/53) of cases demonstrating HPV DNA by PCR linear array. Similarly, Lopes et al.(138) reported a HPV prevalence rate of less than 2% (2/142) in cases of OSCC by quantitative PCR. Scapoli et al.(139) conducted a matched pair case-control analysis using quantitative PCR and ISH to determine the prevalence of HPV in FFPE and fresh frozen samples of OSCC versus matched control tissues. The normal epithelium used as a control and the tumour samples were obtained from the same patient and anatomical region. The authors reported that prevalence of HPV in OSCC was only 2%, and the matched case-control analysis did not demonstrate a significant difference between cases and controls (p=0.37). Based on their findings, the authors concluded that HPV is unlikely to play a significant role in the development of OSCC. Boy et al.(140) observed a prevalence of 12% (7/59) in oral cavity carcinomas, but report HPV 18 to be the only genotype detected by quantitative PCR. Lack of confirmation of PCR findings by ISH suggests the possibility of PCR contamination, a known limitation of this highly sensitive assay (75). A recent large multicentre study by Lingen et al.(79) examined HR HPV in OSCC using p16 immunohistochemistry, ISH, and PCR for HR HPV E6/E7 mRNA. Of 409 cases evaluated 24 (5.9%) were HR HPV positive.

1.4.3 HPV in Oral Epithelial Dysplasia and Oral Potentially Malignant Lesions

Although HPV is implicated in the progression of intraepithelial neoplasia in the cervix (32), less is known about its possible role in the progression of oral lesions (141). In order to further elucidate this relationship, several investigators have examined potentially malignant lesions of the oral cavity for the presence of HPV. As with OSCC, the reported prevalence of HPV DNA in
potentially malignant oral lesions is highly variable, ranging from 0 to 85% (15,62,142). Some of the variability is likely due to case selection, as some studies included leukoplakia and lichen planus as potentially malignant lesions, and others only included epithelial dysplasia (4,143,144). Most of the variability, however, is likely attributable to differences in HPV detection methods (11,15). Therefore, it is perhaps most useful to consider the findings of each study in the context of other studies using similar methodology.

Several studies have found an association of HPV with potentially malignant and malignant oral lesions by PCR (4,62,143,145,146). Campisi et al. (4) reported that the risk of HPV infection was higher in both oral lichen planus (p=0.005) and oral leukoplakia (p=0.01) when compared to normal mucosal controls, using nested PCR and direct DNA sequencing in exfoliated cells. However, HPV 18 rather than HPV 16 was found to be the dominant genotype in this study. The authors suggest that the predominance of HPV 18 over HPV 16 may be due to differences in the geographical origin of samples. Yang et al. (146) reported a HPV prevalence rate of 22.8% in oral leukoplakia, including cases of hyperplasia and varying degrees of dysplasia. Sugiyama et al. (145), found that 36% (16/44) of normal oral mucosa samples, and 61% (31/51) of dysplasias were HPV 16 positive by PCR. There was a statistically significant difference in the rate of HPV detection in dysplasias that progressed to carcinoma compared to those that did not (p= 0.0248). Of the dysplasias that progressed to carcinoma, 82% (18/22) were HPV 16 positive, while only 45% (13/29) of those that did not progress were positive for HPV 16. Although two cases of HPV positive dysplasia developed into HPV negative carcinomas, no HPV positive carcinomas developed from HPV negative dysplasia. Utilizing consensus PCR, PCR-based microarray and p16 immunohistochemistry, Ishibashi et al. (144) reported a HPV prevalence rate of 26.3% for oral premalignant lesions, which included cases ranging from mild dysplasia to carcinoma in situ. Interestingly, however, they did not find that the overexpression of p16 correlated with HPV positivity in premalignant lesions, which suggests that presence of the virus may not be biologically significant.

Ha et al. (75) investigated HPV in premalignant and malignant oral cavity lesions using quantitative PCR to measure viral load. Using a threshold of >1 HPV genome copy/10 cells to signify a productive HPV infection, HPV was detected in only 1 moderate dysplasia (1 of 102 premalignant lesions or 0.98%) and 1 of 34 (2.9%) oral cavity carcinomas.
Angiero et al. (143) utilized nested PCR, catalyzed signal-amplified colorimetric DNA in situ hybridization (CSAC-ISH) and p16 immunohistochemistry to investigate the presence of HPV in oral epithelial dysplasia and oral cancer. Overall, 35.7% (5/14) of moderate/severe dysplasias and 27.3% (3/11) of OSCC cases were HPV positive. Two cases of HPV positive dysplasia contained LR HPV genotypes, while 3 cases had HR HPV genotypes. Of the moderate/severe dysplasia cases considered positive for HR HPV, only one case was positive for both HPV DNA by PCR and ISH, and ISH was not used in one case due to lack of tissue. Of the OSCC cases, 2 showed evidence of LR HPV and 1 showed evidence of HR HPV by PCR and ISH. Interpretation of the significance of this data is limited by a very low number of positive cases, and by the fact that not all diagnostic assays were applied to all cases. Acay et al. (147) utilized ISH with signal amplification to investigate the role of HPV in oral carcinogenesis, and found that 26.7% of leukoplakias with dysplasia demonstrated evidence of HR HPV DNA. Although the prevalence of HPV infection increased marginally with the severity of the lesions, this relationship was not statistically significant. Low risk HPV types 6 and 11 were restricted to cases of no dysplasia or mild dysplasia.

Meta-analyses in this area have not proven to be particularly helpful in providing evidence for definitive conclusions. Miller and Johnstone (148) examined both potentially malignant lesions and carcinomas, and found pooled HPV prevalence rates of 22.2% in benign leukoplakia, 26.2% in intraepithelial neoplasia and 46.5% in SCC, however they did not separately evaluate prevalence rates from the oral cavity and oropharynx. Jayaprakash et al. (141) reviewed the literature to estimate the prevalence of HPV 16/18 in dysplasia of the oral cavity and oropharynx. When considered separately, the prevalence of HPV in oral cavity dysplasia was 25.3%, with no significant difference between anatomic subsites. The prevalence rate for oropharyngeal dysplasia was not reported, as only a very small number of the included studies separated tissue samples by subsite and included tissue from the oropharynx.

It is clear from the literature that studies utilizing non-quantitative PCR consistently report higher rates of HPV infection when compared to those utilizing other methodologies. Further, it is known that the mere presence of HPV DNA in a particular lesion does not necessarily indicate biological or transcriptional activity of the virus (74). Therefore, from a molecular standpoint,
evidence of HPV DNA alone is insufficient to support a causal relationship (79,149). Current research suggests that the use of a single assay for HPV detection may overestimate the rate of biologically significant HPV infections in oral lesions, and it is generally suggested that complementary assays be used to limit false-positive detection rates (82).

In the oral cavity, SCC may be preceded by epithelial dysplasia. This is also true in the uterine cervix where carcinoma is often preceded by cervical intraepithelial neoplasia (CIN). High-risk HPV is detected in CIN and the frequency of detection increases with increasing histologic grade, thus implicating HPV in the progression of CIN to cervical carcinoma (32). It is unclear, however, whether an analogous relationship exists in the oral cavity. While limited evidence suggests that the overall contribution of HPV to oral potentially malignant lesions is likely lower than in the uterine cervix (75,143,144,147), more rigorous studies employing a combined assay approach and precise definition of cases are needed.

1.4.3.1 p16 Expression in Oral Epithelial Dysplasia and Potentially Malignant Lesions

The biology of p16 and its abnormal expression in HPV-related lesions is discussed above (section 1.3.5). Many studies have investigated the role of p16 as a potential biomarker in dysplastic oral mucosal lesions, however results are conflicting. Some of the variability in the reported results may relate to the criteria are used to determine p16 positivity. At present, many studies utilize a quantitative method for assessing p16 positivity. While there is no established cut-off for positivity, >50% of cells stained is one accepted definition (150). Other studies define positivity as diffuse, strong staining, and consider weak or focal staining to be negative, irrespective of the percentage of cells staining (151). Cunningham (151), Gologan (152) and Angiero (153) found that p16 overexpression correlated with increasing grade of dysplasia in the oral cavity. Furthermore, Cunningham found that 6 of 41(14.6%) cases that demonstrated p16 overexpression were also positive for HPV DNA, the majority of which (83%) were type 16. Although Buajeeb et al.(154) did not find p16 overexpression in oral dysplasia, their study was limited by low numbers, and they did not investigate any cases beyond mild dysplasia. Bradley et al. (155) found decreased expression of p16 in dysplastic lesions, with the trend towards absent expression with increasing severity of the lesion. The authors suggest that non-specific staining related to the use of a polyclonal antibody in other studies may have been misconstrued
as overexpression of p16 (155). The current state of the literature concerning the expression of p16 in dysplastic lesions is conflicting. Further, there is inadequate information to determine whether alterations to the expression of p16 (increased or decreased) are predictive of the development of oral SCC (156).

1.4.3.2 Use of MIB-1 in the Evaluation of Oral Epithelial Dysplasia and Potentially Malignant Lesions

Loss of normal epithelial cell maturation is a feature of epithelial dysplasia, and the presence of increased numbers of cycling cells outside the basal compartment, as detectable by Ki-67/MIB-1, theoretically could be used to differentiate between normal and abnormal epithelium (157-159). In this regard, the utility of Ki-67 has been investigated in the study of potentially malignant lesions in the oral cavity. Gonzales-Moles et al. (159) assessed dysplastic and non-dysplastic tissue adjacent to SCC in the oral cavity. They found a significant association between suprabasal expression of Ki-67 and epithelial dysplasia. Angiero and colleagues found that the Ki-67 labelling index increased with the grade of dysplasia, and that localization in the upper 2/3 of the epithelium was significantly associated with moderate or severe dysplasia (153). In a study of malignant progression in oral leukoplakias, Nasser et al. also found that Ki-67 increased with progression to malignancy, but had a poor predictive value, as increased proliferation was also observed in non-progressing, non-dysplastic lesions (160).

1.4.4 Malignant Papillary Lesions

Papillary squamous cell carcinoma (PSCC) is described as a variant of squamous cell carcinoma microscopically characterized by an exophytic or papillary architecture bearing resemblance to squamous papilloma, but with cytologic features of malignancy (161,162). The use of the term PSCC to describe malignant exophytic or papillary lesions in the UADT is controversial, and consensus on PSCC as a distinct clinicopathologic variant of SCC has not been definitively established (163). Disagreement exists over the features needed in order to classify a lesion as PSCC, existence of precursor lesions and distribution over UADT subsites (164-170). Ishiyama and colleagues conducted a study to characterize papillary neoplasms of the upper respiratory tract (165). Cases included in their study showed two major histopathologic patterns: one with an inverting verrucous appearance, prominent keratosis and cytological features of dysplasia, and a second group with an exophytic papillary growth pattern characterized by arborizing papillary
fronds covering fibrovascular connective tissue cores. Thompson et al. (169) also described PSCC as having two main histopathologic patterns, papillary-frond or broad-based exophytic. The exophytic pattern was described to have a more lobulated appearance on cross-section, while the papillary-frond pattern is described as “celery cut across a stalk,” on tangential section. Except for 4 cases, all of the lesions described by Thompson et al. showed some degree of surface keratinization ranging from focal to abundant. Despite Parkhill’s original definition of PSCC as a non-keratinizing lesion, most of the current histopathologic descriptions of PSCC allow for surface keratinization (161,163,169,171).

The larynx is the site most commonly involved by PSCC, followed by the sinonasal tract (162,168), and it is generally thought that PSCC of the larynx and sinonasal tract is a de novo malignancy that does not have an established precursor lesion (169). However, it has also been suggested that PSCC may develop in the site of a previous papilloma (168). Compared with other UADT sites, the incidence of PSCC in the oral cavity is rare, with approximately 70 cases reported in the literature to date (172). Some consider oral papillary squamous neoplasms arising from potentially malignant lesions to fall within the clinical spectrum of proliferative verrucous leukoplakia (PVL). PVL is a distinctive type of high risk leukoplakia which is histologically heterogeneous and has a high rate of malignant transformation (173). The histologic spectrum may include lesions ranging from simple keratosis to flat or verrucous dysplasia, verrucous carcinoma, papillary squamous cell carcinoma and conventional invasive squamous cell carcinoma (165,173). The concept of this relationship between PSCC and PVL was primarily based on observations that transitions from simple keratosis to verrucous keratosis with dysplasia, and PSCC often occurred within synchronous and metachronous leukoplakic lesions (165). Ishiyama et al. (165) found this relationship to be consistent in lesions across different UADT sites including those from the oral cavity, oropharynx and larynx. In contrast, other investigations of PSCC primarily from the larynx and sinonasal tract, emphasize the overall architectural similarities of PSCC to that of a papilloma and generally do not describe a leukoplakic precursor lesion (164). Batsakis et al. strongly believe that PSCC does not occur in the oral cavity and contend that reports of PSCC arising from leukoplakias in the oral cavity represent verrucous or conventional, exophytic SCC arising in a background of PVL (164).
The distinction between PSCC and conventional squamous cell carcinoma may have clinical implications. Thompson et al. (169) found that distinction between exophytic and papillary patterns had prognostic significance in laryngeal PSCC. The authors concluded that both papillary and exophytic PSCC had better outcomes than conventional SCC, and that the papillary subgroup had a better prognosis than the exophytic group. Investigators have also stressed the importance of distinguishing between verrucous carcinoma (VC) and PSCC because of biological and clinicopathologic differences (165). VC is generally thought of as an extremely well differentiated SCC, with bulbous, pushing, acanthotic rete ridges, ‘church spire-like’ hyperkeratosis and the distinct absence of epithelial dysplasia (165,174). Verrucous carcinoma grows slowly and invades with a pushing epithelial front, which is thought to represent direct extension rather than true invasion, and does not metastasize (165). Conversely, PSCC, as described above, is a variant of SCC, which can present as an in situ or invasive lesion (161,165). Ishiyma et al. (165) described multiple instances of nodal metastasis in their series, and concluded that PSCC appeared to behave more aggressively than VC, but overall are less aggressive than conventional SCC, a feature that has been corroborated by other studies (161,169).

Several studies have investigated the role of HPV in PSCC arising in the oropharynx, larynx and sinonasal tracts. Jo et al. found that approximately 68% of papillary squamous cell carcinomas were associated with high-risk HPV infection, most originating in the oropharynx or larynx (166). Thompson et al. (169) investigated laryngeal PSCC and found that only 2.4% (1/41) of cases were positive for HPV 6/11. Suarez et al. (168) reported an overall HPV prevalence rate of 29% (4/14), in which three cases showed LR HPV 6/11 infection, and one case showed HR HPV 16/18 infection. Interestingly, 2 of the 4 HPV positive cases in this study showed evidence of a precursor benign papilloma, and 34% of total cases were associated with a history of previous papilloma at the site of development of PSCC. Conversely, Crissman et al. (161) did not find any evidence of HPV infection by ISH in malignant papillary neoplasms, and no cases were associated with previous recurrent papillomatosis. Although the prevalence of HPV in oral PSCC specifically has not been extensively investigated, there is limited evidence to suggest that HPV is not strongly associated with these lesions. An unpublished study of oral PSCC found HPV in 12% of cases (4 /33) (175). Although Suarez et al. included a small number of oral PSCCs in their series; they did not specify the particular site of the HPV positive PSCCs.
Interestingly, the role of HPV in the development of verrucous carcinoma is controversial (167), as is the prevalence of HPV in proliferative verrucous leukoplakia (164,176-178).

1.4.5 Atypical Papillary Lesions

Occasionally, papillary lesions are encountered that demonstrate cytologic and architectural disturbances. While epithelial atypia and dysplasia has been well documented in respiratory papillomatosis (179-182), the finding of atypia in papillomas and solitary oral papillary lesions is uncommon in immunocompetent persons, and generally not well described. A limited number of studies have investigated the association of HPV with dysplastic and malignant oral verruco-papillary lesions. Stokes et al. (149) examined dysplasias and carcinomas with verrucous or papillomatous morphology, including verrucous carcinoma and well-differentiated SCC with an exophytic verrucous or papillary architecture. They did not find any evidence of high or low risk HPV by ISH despite the demonstration of HPV DNA in some lesions by PCR (1 of 7 carcinomas and 5 of 13 dysplasias). The authors suggest that a low copy number of HPV DNA may account for the negative ISH results, and that presence of viral DNA at low levels likely represents a transient infection rather than transcriptionally active, transforming infection. Hwang et al. (183) also evaluated oral papillary and verrucous lesions for HPV. They evaluated a heterogeneous group of lesions including squamous papillomas, verrucous hyperplasia, squamous hyperplasia and condyloma, some of which contained concomitant malignant foci, and some which transformed to malignancy over time. In contrast to the results of Stokes et al. they found evidence of HPV by ISH in 15.1% (8/53) of cases, including 4 cases of SCC, 1 case of VC and 3 benign lesions. HPV 16 was the most commonly identified subtype, though some lesions showed co-infection with LR and HR HPV types, and one case showed infection with LR HPV only. The authors report a significantly greater incidence of malignant transformation associated with HPV infection (p=0.005). The criteria used to define the different types of verrucal-papillary lesions included in this study are not reported, and photomicrographs are not provided. Malignant transformation of classical squamous papillomas is not described (refer to section 4.1) (105), and thus the high number of squamous papillomas that transformed to SCC in this study (11 cases) contradicts the known behaviour of these lesions. It is possible, therefore, that lesions originally diagnosed as squamous papilloma may have been inadequately sampled or misclassified.
Al-Bakkal et al. (184) investigated HPV 16 E6 expression in exophytic oral lesions in immunocompromised and immunocompetent persons. They found that E6 mRNA was present in a significantly higher proportion of exophytic lesions exhibiting premalignant and malignant features (72%) when compared to benign exophytic lesions (34%, p=0.0008). This relationship was stronger in immunosuppressed (92%) versus immunocompetent patients (65%). In HIV positive patients, HPV has been identified in papillary lesions with cytologic atypia. Although the significance of these changes is unclear, it is thought that they might represent a stage of premalignancy (185). Long-term follow-up of histologically dysplastic oral warts in HIV positive patients did not show progression to SCC (186). Regezi et al. (186) speculate that the rate of progression to SCC is low because certain invasion associated proteins may be underexpressed in dysplastic warts in HIV infected patients. The natural history of dysplastic papillary lesions is currently unknown; however, preliminary investigations suggest that transformation rates are low. Although primarily affected by LR HPV types 6/11 rather than HR HPV types, most cases of recurrent laryngeal papillomatosis with dysplasia do not progress to malignancy (161). The mechanisms underlying rare cases of malignant transformation in laryngeal papillomatosis are not well understood (161).

1.4.5.1 Ki-67 in Oral Papillary Lesions

Regezi et al. (186) investigated Ki-67 expression in dysplastic and non-dysplastic warts, and SCC. They reported markedly higher levels of Ki-67 expression in both dysplastic warts and SCC as compared to non dysplastic warts and normal epithelium. They speculate that this increased proliferation may be the result of HPV infection, as all the dysplastic warts were from individuals infected by HIV, a population known to be at increased risk for the development of HPV related lesions (185). Interestingly, however, neither increased proliferative fraction, nor dysplasia was predictive of malignant transformation (186).

1.5 Oral HPV Infection as a Risk Factor for Head and Neck Squamous Cell Carcinoma

A number of studies have evaluated the potential relationship between oral HPV infection and head and neck cancer risk. Oral HPV infection has been measured by analyses of buccal
scrapings, saliva or oral rinse samples for HPV. Most studies employ PCR or nested PCR techniques to detect HPV DNA. By examining rates of oral HPV infection in individuals with known HNSCC in a cohort, or by comparison with a control population in case-control studies, investigators have demonstrated an association between oral HPV infection and HPV positive SCC. Agrawal et al. (187) utilized serial oral rinse samples to test for oral HPV infection in patients with HPV 16 positive and negative HNSCC over 3 years. The authors found that high risk oral HPV infections were more frequent in individuals with HPV 16 positive tumours prior to, and after therapy than those with HPV negative carcinoma. Further, the HPV DNA from tumour samples in patients with positive oral rinse samples was identical in most cases (91% of baseline and 60% of follow-up samples), which the authors attribute to the presence of exfoliated tumour cells captured with the rinse. In another study utilizing oral rinse samples, Smith et al. (188) found that HR HPV types were detected in samples from 22.9% of patients with HNSCC and 10.8% of control patients that did not have carcinoma. Additionally, patients with HR HPV positive tumours were more likely to demonstrate HR HPV in their oral rinse samples when compared with individuals with HPV negative tumours (57.9% vs. 15.1%), and this relationship was statistically significant. HPV DNA was detected in 15% of oral cavity cancers and 38% of oropharyngeal cancers, and patients with oropharyngeal cancer were found to be more likely to have HR HPV in exfoliated cells as compared to those with oral cavity cancers (OR=3.6, 95% CI 1.8-7.1). Similarly, D’Souza et al. (137) found that oropharyngeal carcinoma was significantly associated with oral HPV 16 infection or oral HPV infection of any type. Gillison et al. also reported that the presence of oral HPV 16 infection was strongly associated with HPV 16 positive head and neck squamous cell carcinoma, and a 50 fold greater risk for developing oropharyngeal squamous cell carcinoma, based on results from case-control studies (52,124). Fakhry et al.(189) analyzed oral rinses and oropharyngeal brush biopsies (Oral CDX brush) for HPV infection using two techniques: PCR linear array for multiple HPV types and quantitative PCR specific for HPV 16 to measure viral load. Two patient populations were studied: patients with clinically demonstrable abnormalities of the oropharynx, and HIV positive individuals known to be at increased risk for oral HPV infection and oropharyngeal squamous cell carcinoma. In individuals with clinical lesions, the authors report that HPV 16 DNA and abnormal cytology were strongly associated with carcinoma. However, in the ‘at risk’ group of patients that did not have clinically detectable lesions, tonsillar HPV 16 infection was not associated with cytologic changes detectable by brush biopsy.
Thus, though these studies support the concept that oral infection with HR HPV may place individuals at increased risk for oropharyngeal carcinoma, whether the presence of oral infection is predictive for the development of pre-cancer or the progression of established cancer, is unknown (188). Many of these studies utilized PCR based HPV DNA detection methods, which cannot distinguish between transient and potentially oncogenic infections, limiting their clinical value. Further, as important information about the natural history of oral HPV infection, such as the rate of viral clearance, and the influence of factors such as age, sex and smoking status are largely still unknown, the clinical utility of repeated testing (51), or any testing, in the general population is questionable.
Chapter 2. Statement of Objectives and Hypotheses

2.1 Objectives

1. Determine the frequency of clinically relevant HPV infection in oral epithelial dysplasia and atypical and malignant oral papillary lesions

2. Correlate the finding of low and high risk HPV in oral lesions with clinical and pathologic features and clinical outcome

3. Study a series of atypical and malignant papillary lesions with respect to clinical and histologic features and the presence of HPV, and compare these lesions with conventional squamous papillomas

2.2 Hypotheses

1. Clinically relevant, as opposed to transient infection by HPV can be demonstrated by ISH and associated with histopathologic findings consistent with the biologic effects of HPV

2. High risk HPV is associated with a subset of oral epithelial dysplasias

3. Atypical papillary lesions are a heterogeneous group of lesions with respect to the presence of HPV and clinical behavior

4. The majority of malignant papillary lesions are not associated with high risk HPV infection
Chapter 3. Association of High-risk Human Papillomavirus Infection with Oral Epithelial Dysplasia


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Abbreviations - HPV - Human Papillomavirus; OSCC - oral squamous cell carcinoma; CIN - cervical intraepithelial neoplasia; OED - oral epithelial dysplasia; OPDS - oral pathology diagnostic service; IHC - immunohistochemical staining; ISH - in situ hybridization; PCR - polymerase chain reaction
3.1 Abstract

Objective. The aim of this study was to evaluate cases of oral epithelial dysplasia for biologically significant HPV infection.

Study Design. 40 consecutive cases of high-grade dysplasia and 37 cases of low-grade dysplasia were examined for p16\textsuperscript{INK4a} expression by immunohistochemistry. High risk HPV infection was assessed in p16 positive cases using in-situ hybridization. Proliferation index was assessed with MIB-1 immunohistochemistry.

Results. 11 of 40 high-grade dysplasias and 1 of 37 low-grade dysplasias were p16 positive. High risk HPV was detected in seven cases of p16 positive high-grade dysplasia. The difference between high and low grade dysplasia was statistically significant (p=0.01). HPV positive high-grade dysplasias showed a distinctive histologic appearance and MIB-1 labelling pattern. The majority of high risk HPV-positive cases were seen in the floor of mouth.

Conclusion. High risk HPV was associated with a subset of cases of severe epithelial dysplasia/carcinoma-in-situ that demonstrated diffuse loss of squamous differentiation and a high proliferation index.

Statement of Clinical Relevance

This study demonstrates that high risk HPV infection can be found in a small number of high grade oral epithelial dysplasias, and that the floor of the mouth may be a relatively frequent site for oral HPV infection.
### 3.2 Introduction

Human papillomaviruses (HPV) are epitheliotropic, double stranded DNA viruses that cause lesions in the skin, anogenital and upper aerodigestive tract mucosa. More than 100 types of HPV have been reported and these are stratified into high and low risk groups based on molecular and epidemiologic evidence of association with carcinoma of the uterine cervix (1,2). It is now established that high risk HPV is also associated with squamous cell carcinoma of the oropharynx, particularly the tonsils and base of tongue. Recent studies have shown that 62 - 66% of oropharyngeal carcinomas are HPV positive (3,4). HPV-positive oropharyngeal carcinomas are distinct from HPV-negative carcinomas in their epidemiology, histopathology, molecular pathology and response to treatment. HPV-positive carcinomas have a basaloid, non-keratinizing histomorphology and show overexpression of the cell cycle regulating protein p16INK4A. Compared with HPV-negative oropharyngeal carcinomas, HPV-positive tumors show higher response rates to chemoradiation therapy leading to better survival of the patients (5-7).

The squamous mucosa of the oral cavity has similarities in structure to both uterine cervical mucosa and oropharyngeal mucosa and is susceptible to HPV infection (8). Recent reports have indicated a low prevalence of HPV (4 – 6%) in squamous cell carcinoma of the oral cavity, in contrast to the oropharynx (9,10). Oral squamous cell carcinomas (OSCC) may be preceded by epithelial dysplasias which are graded according to the extent of histologic abnormalities within the epithelium, analogous to cervical intraepithelial neoplasia (CIN). High-risk HPV is detected in CIN and the frequency of detection increases with increasing histologic grade, thus implicating HPV in the progression of CIN to cervical carcinoma (11). Reports of HPV in oral epithelial dysplasias have not been consistent, partly because of differences in case selection and methods used to detect HPV (12,13).

This study examined cases of oral epithelial dysplasia (OED) for biologically significant oncogenic HPV infection, using currently recommended methods of detection (14,15). In view of the association of high-risk HPV with high-grade CIN of the uterine mucosa, we hypothesized that HPV would be detected predominantly in high-grade OED which included severe dysplasia and carcinoma-in-situ, and much less in low-grade OED which included mild and moderate dysplasia (16). We also examined the relationship between HPV detection and clinical parameters of patient age and sex, and oral cavity subsite.
3.3 Materials and Methods

3.3.1 Tissue Samples

This was a retrospective study of biopsy specimens of oral mucosal lesions submitted to the Oral Pathology Diagnostic Service (OPDS) at the Faculty of Dentistry, University of Toronto, by general and specialist dentists in the province of Ontario. This study was approved by the Research Ethics Board of the University of Toronto (protocol #26571). A search of the OPDS files from 2007 to 2009 inclusive for cases with a diagnosis of severe epithelial dysplasia or carcinoma-in-situ yielded 40 cases of high-grade dysplasia with sufficient tissue in the paraffin block for analyses. For comparison with the high-grade dysplasias, consecutive cases of mild or moderate dysplasia were retrieved from the same period to give a group of 37 low-grade dysplasias that were matched to the group of high-grade dysplasias for age and sex of the patients and for site of lesion (Table 3.1). None of the patients had a history of immunosuppression. Ten cases of normal oral epithelium from either fibroepithelial polyp or amalgam tattoo taken from a variety of oral sites were used as normal controls.

| Table 3.1 Clinical and pathologic characteristics of dysplasia cases |
|---------------------------------|-----------------|-----------------|-----------------|
|                                 | Total           | High grade dysplasia | Low grade dysplasia |
| Number of cases                 | 77              | 40               | 37              |
| Age (median and range)          | 58 (15-84)      | 59 (15-84)       | 58 (29-83)      |
| Sex                             | Male            | Female           |
|                                 | 46              | 31               |
|                                 | 23              | 17               |
|                                 | 23              | 14               |
| Site                            | Floor of mouth  | Tongue           | Buccal mucosa   |
|                                 | 29              | 32               | 3               |
|                                 | 16              | 16               | 2               |
|                                 | 13              | 16               | 1               |
|                                 | Gingiva         | Hard palate      |
|                                 | 8               | 5                | 4               |
|                                 | 3               | 1                | 4               |
3.3.2 Immunohistochemical Staining

Immunohistochemical staining (IHC) for p16 protein was performed on 4 µm thick sections of formalin-fixed, paraffin-embedded biopsy specimens, using a monoclonal antibody against p16 (CINtec, Roche mtm laboratories) and a Ventana Benchmark automated slide stainer according to manufacturer’s instructions (Ventana Medical Systems, Inc., Tucson, AZ, USA). A case of HPV-positive oropharyngeal carcinoma was used as the positive staining control. Cases of normal oral epithelium were used as negative staining controls. Both nuclear and cytoplasmic staining for p16 protein was seen in epithelial cells, in agreement with previous reports (14,17). p16 staining was scored as positive (grade 2) when there was strong, diffuse staining of dysplastic epithelium involving at least the deep half of the epithelial thickness. Staining was scored as equivocal (grade 1) when there was patchy staining with unstained epithelial cells interspersed among positive cells. Cases with no staining or staining of scattered, single cells within the dysplastic epithelium were scored as negative (grade 0). All cases were scored by two observers (C.M. and G.B.) following the criteria above.

Immunohistochemical staining for Ki-67 antigen was performed with the monoclonal antibody MIB-1 (Dako Canada, Burlington, Ontario). Normal oral epithelium was used as the control, where a subset of cells in the basal and parabasal layer showed staining of the nucleus (grade 1). Staining of a subset of basal, parabasal and suprabasal cells extending to half the thickness of the epithelium was scored as grade 2. Staining extending into the superficial half but not all epithelial layers was scored as grade 3. Staining of full thickness of the epithelium up to the keratinized surface cells was scored as grade 4.

3.3.3 In situ DNA hybridization for High and Low Risk HPV

In situ hybridization (ISH) for HPV DNA was performed on 4 µm thick sections of formalin-fixed, paraffin-embedded biopsy specimens, using the Inform HPV III Family 16 probe (B) for high risk HPV and the Inform HPV II Family 6 probe (B) for low risk HPV on a Ventana Benchmark automated slide stainer according to manufacturer’s instructions (Ventana Medical Systems, Inc., Tucson, AZ, USA). The Family 16 probe detected high risk HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 66. The Family 6 probe detected low risk HPV types 6 and 11. High risk HPV 3-in-1 control slides supplied by the manufacturer were used, including CaSki cells (HPV16 positive, 200-600 copies per cell), HeLa cells (HPV18 positive, 10 - 50 copies per
cell) and T24 cells (HPV negative). In addition, a case of HPV16 positive oropharyngeal carcinoma was used as positive control for the Family 16 probe. A case of condyloma acuminatum was used as the positive control for the Family 6 probe and normal oral epithelium was used as the negative staining control. ISH for HPV DNA was scored as positive when blue reaction product was seen in the nucleus of dysplastic epithelial cells, in either a homogeneous or punctate pattern. Staining that was not within the nucleus of epithelial cells was interpreted as background staining. All cases were scored by two observers (C.M. and G.B.) following the criteria above.

3.3.4 Statistical Analysis

Descriptive statistics were provided as median and range for continuous variables and frequencies and proportions for categorical variables. The association between HPV infection status and clinicopathologic variables was analyzed in contingency tables, using the Fisher exact test and the Wilcoxon rank-sum test. Two-sided tests were applied. Results were considered significant if the p-value was less than or equal to 0.05. All the statistical analyses were performed with SAS 9.2 (SAS Institute, Cary, NC, USA).

3.4 Results

3.4.1 Immunohistochemical Staining for p16 Protein

Strong, diffuse staining for p16 was observed in 11 of 40 (27.5%) cases of high grade dysplasia, and 1 of 37 (2.7%) cases of low grade dysplasia. These cases (grade 2 staining) were characterized by diffuse positive staining of the dysplastic epithelium, with staining of both nucleus and cytoplasm. The staining involved at least the deep half of the epithelial thickness in all cases of high grade dysplasia (Figure 3.1B). The case of low grade dysplasia showed diffuse staining of the basal and parabasal layers (Figure 3.2B). Non-dysplastic epithelium that was adjacent to the dysplastic epithelium did not show p16 staining (Figure 3.1B).

In 1 of 40 (2.5%) cases of high grade dysplasia and 7 of 37 (18.9%) cases of low grade dysplasia, there was patchy p16 staining where groups of dysplastic epithelial cells showed nuclear and cytoplasmic staining with unstained epithelial cells interspersed among the positive cells (grade 1
staining). The groups of p16 positive cells were either located in the basal and parabasal epithelium or restricted to the suprabasal layers with no staining of basal cells (Figure 3.3B).

The remaining cases of dysplasia showed either no staining for p16, or scattered epithelial cells with weak staining, and were classified as p16 negative (grade 0 staining) (Figure 3.4B).

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**Figure 3.1.** A, Hematoxylin and eosin stained section of p16 positive, HPV positive, high grade dysplasia. B, High grade dysplasia with strong, diffuse immunohistochemical staining for p16 in the dysplastic epithelium but not in the adjacent epithelium. C, ISH with probe for high risk HPV showing punctate and homogenous staining of epithelial nuclei; inset shows details of hybridization signal that is localized to the nuclei. D, MIB 1 immunohistochemical staining shows a high labeling index with positive nuclei throughout the basal and suprabasal epithelium up to the parakeratinized layers. (Magnification x50; inset to C x400)
Figure 3.2. A, Hematoxylin and eosin stained section of p16 positive, HPV negative, low grade dysplasia. B, Low grade dysplasia with strong, diffuse immunohistochemical staining for p16 in the basal and parabasal layers of the entire dysplastic lesion. C, ISH of the same case shows no evidence of high risk HPV. D, MIB 1 immunohistochemical staining shows positive nuclei in the basal and parabasal layers. (Magnification x50)
Figure 3.3. A, Hematoxylin and eosin stained section of low grade epithelial dysplasia. B, Low grade dysplasia with patchy immunohistochemical staining for p16. C, ISH of the same case shows no evidence of high risk HPV. (Magnification x50)

Figure 3.4. A, Hematoxylin and eosin stained section of high grade dysplasia. B, Same case with no significant p16 staining. C, MIB1 immunohistochemical staining shows positive nuclei scattered within the deep half of the epithelium. (Magnification x50)
3.4.2 In situ Hybridization for HPV DNA

All cases with strong diffuse (grade 2) and patchy (grade 1) p16 staining were examined with ISH for high risk HPV. Seven of the 11 cases of high grade dysplasia that exhibited strong, diffuse p16 staining showed high risk HPV DNA in the dysplastic epithelium. The labelled nuclei were mostly located in the upper spinous layers, and groups of labelled nuclei were present in focal areas within the dysplastic lesion rather than throughout the lesion (Figure 3.1C). Both homogeneous and punctate patterns of labelling were observed, sometimes within a single lesion, indicating both episomal and integrated viral DNA. The case of low grade dysplasia with strong, diffuse staining for p16, and all cases of dysplasia with patchy p16 staining failed to show evidence of high risk HPV (Figure 3.2C, 3.3C). Overall, 7 of 77 cases of dysplasia were p16 positive and high risk HPV positive.

The 5 cases of dysplasia with strong, diffuse p16 staining but no evidence of high risk HPV infection were studied further by ISH with a probe for low risk HPV (types 6 and 11). In addition, 5 cases of dysplasia with patchy p16 staining were also examined for low risk HPV. The 3 remaining cases with patchy p16 staining could not be studied further because of lack of tissue. None of the dysplasias showed evidence of low risk HPV DNA in our analyses. The results of ISH for high and low risk HPV are presented in Table 3.2.
Table 3.2  HPV status in relation to p16 expression in cases of epithelial dysplasia

<table>
<thead>
<tr>
<th>Case #</th>
<th>Grade of dysplasia</th>
<th>p16 staining score</th>
<th>p16 staining pattern</th>
<th>High risk HPV by ISH</th>
<th>Low risk HPV by ISH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>high</td>
<td>2</td>
<td>strong, diffuse</td>
<td>Pos</td>
<td>n.d.</td>
</tr>
<tr>
<td>3</td>
<td>high</td>
<td>2</td>
<td>strong, diffuse</td>
<td>Pos</td>
<td>n.d.</td>
</tr>
<tr>
<td>9</td>
<td>high</td>
<td>2</td>
<td>strong, diffuse</td>
<td>Pos</td>
<td>n.d.</td>
</tr>
<tr>
<td>11</td>
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<td>strong, diffuse</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>16</td>
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<td>1</td>
<td>patchy</td>
<td>Neg</td>
<td>n.d.</td>
</tr>
<tr>
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<td>strong, diffuse</td>
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<td>Neg</td>
</tr>
<tr>
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<td>Neg</td>
</tr>
<tr>
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<td>strong, diffuse</td>
<td>Pos</td>
<td>n.d.</td>
</tr>
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<td>Neg</td>
</tr>
<tr>
<td>38</td>
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<td>2</td>
<td>strong, diffuse</td>
<td>Pos</td>
<td>n.d.</td>
</tr>
<tr>
<td>40</td>
<td>high</td>
<td>2</td>
<td>strong, diffuse</td>
<td>Pos</td>
<td>n.d.</td>
</tr>
<tr>
<td>46</td>
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<td>1</td>
<td>patchy</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>47</td>
<td>low</td>
<td>1</td>
<td>patchy</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>48</td>
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<td>1</td>
<td>patchy</td>
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<td>Neg</td>
</tr>
<tr>
<td>49</td>
<td>low</td>
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<td>patchy</td>
<td>Neg</td>
<td>n.d.</td>
</tr>
<tr>
<td>50</td>
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<td>patchy</td>
<td>Neg</td>
<td>n.d.</td>
</tr>
<tr>
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</tr>
<tr>
<td>71</td>
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<td>1</td>
<td>patchy</td>
<td>Neg</td>
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</tr>
</tbody>
</table>

n.d., not done.

3.4.3  Clinical and Pathologic Features of HPV Positive Dysplasias

Five of the 7 HPV positive cases were floor of mouth lesions, one was from the lingual mandibular mucosa, and one from the anterior hard palate (incisive papilla area). However, the association between high risk HPV infection and site of the dysplastic lesion did not reach statistical significance (Table 3.3).

All HPV positive cases were high grade dysplasias. There was a statistically significant association between high risk HPV infection and high grade dysplasia (Table 3.3).
The histomorphology of the dysplasia cases was reviewed in H&E sections. As expected, all cases with the diagnosis of severe dysplasia and carcinoma-in-situ shared the common feature of at least focal areas with cytological atypia and loss of maturation affecting more than two-thirds of the thickness of the stratified squamous epithelium. Within this general framework, there was variation in histologic appearance among the high grade dysplasias that correlated with high risk HPV status. The HPV-positive dysplasias could be distinguished from the HPV-negative cases, because they showed loss of squamous differentiation and a basaloid appearance throughout the dysplastic lesion (Figure 3.1A), rather than in a focal or segmental pattern (Figure 3.4A). In the low grade dysplasias, cytological atypia and irregular maturation were restricted to the deeper epithelial layers, affecting less than two-third of the epithelial thickness (Figures 3.2A, 3.3A).

Abnormal proliferation of the dysplastic epithelium was studied by immunohistochemical staining for the Ki-67 antigen, using the MIB-1 antibody. The MIB-1 labelling pattern corroborated the histomorphological difference between HPV-positive and HPV-negative high grade dysplasias. HPV-positive, high grade dysplasias were characterized by MIB-1 stained nuclei throughout the basal and suprabasal layers, staining at least two-thirds of the thickness of the epithelium. In some cases, MIB-1 stained nuclei were seen in the entire epithelium up to the parakeratinized surface layers (Figure 3.1D). In the HPV-negative, high grade dysplasias, the MIB-1 staining pattern was variable. In many of these cases, positive nuclei were confined to the deep half of the epithelium (Figure 3.4C). The low grade dysplasias showed MIB-1 staining of basal and parabasal nuclei, and no staining of the suprabasal cells (Figure 3.2D).
Table 3.3 High risk HPV status in relation to patient age and sex, site of lesion and grade of dysplasia

<table>
<thead>
<tr>
<th>Clinicopathologic variable</th>
<th>All dysplasia (n=77)</th>
<th>HPV positive (n=7)</th>
<th>HPV negative (n=70)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (range)</td>
<td>58 (15-84)</td>
<td>50 (29-69)</td>
<td>58.5 (15-84)</td>
<td>0.24</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>0.70</td>
</tr>
<tr>
<td>Female</td>
<td>31</td>
<td>2</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>Site</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Floor of mouth</td>
<td>29</td>
<td>5</td>
<td>24</td>
<td>0.056</td>
</tr>
<tr>
<td>Tongue</td>
<td>32</td>
<td>0</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>Other (gingiva, buccal mucosa, hard palate)</td>
<td>16</td>
<td>2</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>Grade of dysplasia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low grade</td>
<td>37</td>
<td>0</td>
<td>37</td>
<td>0.01*</td>
</tr>
<tr>
<td>High grade</td>
<td>40</td>
<td>7</td>
<td>33</td>
<td></td>
</tr>
</tbody>
</table>

HPV status in relation to patient sex, site of lesion and grade of dysplasia were analyzed by Fisher’s exact test; relation with patient age was analyzed by Wilcoxon test; * indicates statistically significant difference between HPV positive and negative, p<0.05.
3.5 Discussion

This study has shown that a small subset of oral epithelial dysplasias (OED) was associated with high risk HPV infection. These HPV-positive dysplastic lesions had a characteristic histologic appearance and Ki-67 labelling pattern which demonstrated a diffuse, full-thickness loss of squamous differentiation and abnormal proliferation, consistent with the oncogenic effects of high risk HPV. The floor of mouth appeared to be the predominant oral site for HPV associated dysplastic lesions, presumably because of its thin, non-keratinized epithelium. A greater thickness of epithelium and/or keratinization at other oral sites may provide a barrier to prevent the virus from gaining access to the basal layer of stratified squamous epithelium to initiate infection (18).

Previous reports of the frequency of HPV infection in OED have been highly variable (12,13). The variability could be due to differences in case selection and in methods used to detect HPV. Studies that included lesions from the area of Waldeyer’s ring (base of tongue, palatine tonsils and soft palate) reported a higher proportion of HPV-positive cases (19). Studies that included lesions from immunocompromised patients found a higher frequency of HPV infection, and infection with multiple HPV genotypes (20). There were also studies that included oral epithelial dysplasia within a larger, clinically defined group of oral potentially malignant disorders (13,21). Different methods have been used to detect HPV. These include polymerase chain reaction (PCR) with HPV generic primers in combination with viral DNA sequencing or hybridization to type-specific probes (22,23), quantitative PCR (24), and in-situ hybridization to viral DNA (25,26) . PCR-based detection methods that use HPV generic primers are highly sensitive, but may not discriminate between biologically significant infection and the incidental presence of HPV at low copy numbers (24,27,28).

We conducted a retrospective study of epithelial dysplasia from the oral cavity proper, excluding Waldeyer’s ring, in patients with no history of immunosuppression. The use of archival formalin-fixed, paraffin embedded material and well established, proprietary systems for p16 staining and HPV detection should facilitate future comparison with studies in other centres.
Our study demonstrated that high risk HPV was associated with a subset of OED and that there was a clear correlation between HPV detection and histomorphology. All 7 HPV positive dysplasias exhibited diffuse, full-thickness loss of squamous differentiation (Figure 1A). Mitotic figures and mitotic-like structures, multinucleated cells and dyskeratotic cells were seen throughout the thickness of the epithelium. These histologic findings were previously described for a subset of oral bowenoid lesions that were considered to be analogous to Bowen’s disease of the skin and were positive for HPV16/18 (29). The HPV positive cases of OED that were identified in our study did not fit the criteria for koilocytic dysplasia, which was described as a variant of OED characterized by the presence of koilocytes and associated with a high prevalence of infection by HPV 16/18 and/or 31/33/51 (20). Koilocytic dysplasia shows a combination of superficially located koilocytic cells and mild, moderate or severe dysplasia. In contrast, the 7 HPV positive dysplasias in our study showed severe dysplasia or carcinoma-in-situ throughout the lesion, but koilocytes could not be identified consistently. An association between a basaloid histomorphology and infection with high risk HPV was also reported in a study of oral neoplasms and dysplasias with basaloid differentiation of epithelial cells (30). Among 7 cases of hyperplasia/dysplasia with basaloid epithelial cells, 2 cases were positive for high risk HPV, but there were no illustrations of the histomorphology of these cases (30).

Immunohistochemical staining for p16 has been reported to be a highly sensitive marker for high risk HPV infection of mucosa (17,31). p16 is the major inhibitor of phosphorylation of pRb by cyclin dependent kinases 4/6. Disruption of the pRb regulatory pathway by the HPV E7 oncogene results in overexpression of p16 in a diffuse pattern throughout the HPV infected dysplastic lesion. In contrast, cases of mucosal dysplasia and carcinoma not infected with HPV show loss of p16 expression as a result of hypermethylation, deletion or mutation of the p16 gene. We used p16 staining as a screening tool to identify HPV positive dysplastic lesions. p16 staining of dysplastic epithelium was seen in either a diffuse, continuous or patchy, discontinuous pattern (Figures 1B, 3B). Further investigation of p16-positive dysplastic lesions by ISH demonstrated high risk HPV DNA only among dysplastic lesions with diffuse, continuous p16 staining (Table 3.2).

The high risk HPV probe that was used for ISH analysis recognizes multiple HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 66), therefore it is not clear which of these HPV types were present in our cases of OED. However, previous reports of high risk HPV in head and neck
carcinomas have indicated that HPV 16 was detected in at least 90% of cases, while HPV 18, 31 and 33 were much less commonly seen (32,33).

The finding of HPV DNA per se is not sufficient evidence for clinically significant infection, and demonstration of E6 and/or E7 viral oncogene expression is considered to be a requirement for establishing a causative role of HPV in epithelial pathology (9,34). PCR-based methods to detect mRNA expression of the E6/E7 oncogenes have been used in a small number of studies to provide conclusive evidence for clinically relevant HPV infection in head and neck carcinomas. There are difficulties with mRNA analyses in routinely processed, formalin-fixed paraffin-embedded mucosal biopsies, because of RNA degradation and mixture of mRNA extracted from lesional and non-lesional tissues resulting in decreased reliability and sensitivity of the assay (15,27). p16 immunohistochemical staining can be used as an alternative, albeit indirect method to indicate E7 viral oncogene expression, through a mechanism in which E7 inactivates pRb, and releases the negative feedback inhibition on p16 expression (18,28,35). In our study, positive ISH for high risk HPV was always associated with strong, diffuse p16 staining of the dysplastic epithelium, which provides indirect evidence of E7 oncogene activity.

In the present study, ISH revealed high risk HPV DNA within the nuclei of upper spinous cells of dysplastic epithelial lesions, in both homogeneous and punctate patterns, which were interpreted as episomal and integrated viral DNA respectively. Integration of the HPV genome into the host genome leads to changes in viral gene expression, particularly an increase in expression of the E6 and E7 oncogenes. The degree of viral integration that is necessary for abnormal epithelial proliferation resulting in epithelial dysplasia and carcinoma is still under investigation (11).

In consideration of our finding of high risk HPV DNA that was localized to the nuclei of epithelial cells in dysplastic lesions, and the association of HPV detection with a diffuse pattern of abnormal, increased proliferation and loss of squamous differentiation of the mucosal epithelium, we conclude that there is evidence of clinically significant infection of the oral mucosa by oncogenic HPV in a small number of cases of OED. Interestingly, the high risk HPV associated lesions in our study were predominantly flat or mildly corrugated at the surface, and lacked a papillary or verrucous growth pattern. This is analogous to HPV infection of the uterine
cervical mucosa, where high grade squamous intraepithelial lesions are associated with high risk HPV while condylomas are associated with low risk HPV (11).

Our results are in agreement with a previous study that described strong, diffuse p16 staining of dysplastic epithelium as a marker for high risk HPV infection in high grade OED (36). However, 4 of our 11 cases of high grade dysplasia with strong, diffuse p16 staining did not show evidence of high or low risk HPV infection by ISH (Table 3.2). It is possible that these lesions contain HPV at a copy number that is below the detection limit of the ISH assay. Overexpression of p16 protein may also occur through an HPV-independent mechanism such as senescence of epithelial cells. However, the Ki-67 labelling pattern seen in these cases was not consistent with senescence. Our data on p16 protein expression indicate that epithelial dysplasias with strong, diffuse p16 staining should be further analyzed with a direct assay for HPV, preferably with ISH on serial sections.

3.6 Conclusion

Our retrospective review of archival, formalin-fixed, paraffin-embedded biopsies of OED has shown that high risk HPV was associated with a subset of severe epithelial dysplasia/carcinoma-in-situ characterized by diffuse loss of squamous differentiation and a high proliferation index throughout the basal and suprabasal epithelial layers. High risk HPV-associated OED can be accurately identified in diagnostic oral pathology laboratories by p16 immunohistochemical staining followed by ISH with probes for HPV DNA. This approach should facilitate large scale studies to compare the behaviour of HPV-positive and HPV-negative OED and their respective roles in OSCC development.

3.7 Disclosures and Acknowledgements

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The authors acknowledge the support of Ventana Medical Systems in making the Inform HPV II and III probes available.

The authors report no conflicts of interest related to this study.
3.8 References


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Chapter 4. Association of Human Papillomavirus with Atypical and Malignant Papillary Oral Lesions

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**Abbreviations** – HPV – Human Papillomavirus; IHC – Immunohistochemical staining; ISH – in situ hybridization; PCR – polymerase chain reaction
4.1 Abstract

Objective. To examine atypical and malignant papillary oral lesions for low and high risk HPV infection, and to correlate HPV infection with clinical and pathologic features.

Study Design. Twenty-eight atypical papillary lesions (APLs) and 14 malignant papillary lesions were examined for low risk HPV by in situ hybridization, and p16\textsuperscript{INK4a} and MIB-1 by immunohistochemistry. Cases that showed p16 staining were investigated for high risk HPV. Twenty-four conventional papillomas were studied for comparison.

Results. Low risk HPV was demonstrated in 10 of 66 cases, including 9 APLs and 1 papilloma. Low risk HPV positive cases showed suprabasilar MIB-1 staining, and the agreement was statistically significant (p<0.0001). Diffuse p16 staining and high risk HPV was not seen in any of the cases. A subset of HPV-negative APLs progressed to carcinoma.

Conclusion. Oral papillary lesions are a heterogeneous group. Low risk HPV infection is associated with a small number of benign papillary lesions with virally induced atypia. Potentially malignant atypical papillary lesions and malignant papillary lesions are not associated with low or high risk HPV.
4.2 Introduction

Papillary lesions of the oral mucosa present with a range of clinical and histologic appearances from benign to malignant. Squamous papilloma is a common and benign lesion that is clinically well-demarcated, round or oval in shape, and generally less than 1 cm in diameter. Recurrence is rare after conservative excision. Histologically, the papillary projections are covered by acanthotic stratified squamous epithelium with basilar hyperplasia and a normal pattern of maturation of the squamous epithelium (1). Less frequently, there are lesions that architecturally resemble a papilloma, but with clinically or histologically atypical features. Clinical atypia includes larger size, irregular outline, progressive growth and recurrence. Histologically, these lesions show variably shaped rete ridges and dysplasia affecting the deep epithelial layers. Oral warts with epithelial atypia have been described in HIV-positive patients (2). However, atypical papillary lesions (APLs) of the oral mucosa in immunocompetent persons have not been well documented with respect to diagnostic criteria and biologic potential. The term verrucous is commonly used as a synonym of papillary to describe lesions with surface projections.

Verrucous hyperplasia refers to exophytic, proliferative epithelial lesions with either sharp or blunt surface projections. Verrucous hyperplasia may be clinically indistinguishable from verrucous carcinoma and is thought to be its precursor lesion. The distinction between verrucous hyperplasia and verrucous carcinoma is made histologically (3). Verrucous hyperplasia and carcinoma are closely associated with the clinicopathological diagnosis of proliferative verrucous leukoplakia (4,5).

The nomenclature of malignant papillary lesions (MPLs) is also controversial and the term may include papillary carcinoma, papillary squamous cell carcinoma and exophytic squamous cell carcinoma (6-9). Papillary carcinomas have been defined as exophytic epithelial proliferations that may have features of carcinoma in situ, or may have pronounced nuclear and cellular pleomorphism in the deeper epithelium with keratinization at the surface (6). Other authors have characterized the epithelium of papillary squamous cell carcinoma as high grade dysplasia with no surface keratosis (8). There seems to be agreement that invasion in papillary carcinoma is usually superficial and may be difficult to demonstrate unequivocally (8,9). These lesions are
considered distinct on one hand from conventional squamous cell carcinoma with both exophytic and endophytic, invasive components, and on the other hand from verrucous carcinoma (10).

Human Papillomavirus (HPV) has been shown to cause a variety of papillary lesions of skin and mucosa, including verruca vulgaris, condyloma acuminatum, focal epithelial hyperplasia and recurrent respiratory papillomatosis (11). Previous studies have examined benign, atypical and malignant oral papillary lesions for the presence of HPV, using a variety of methods, including immunohistochemical staining for HPV common antigen, in situ hybridization (ISH) with DNA probes, polymerase chain reaction (PCR) and in situ reverse transcriptase PCR. The reported prevalence of HPV in oral squamous papillomas varies from 13% to 68% and all positive cases are low risk HPV types 6/11 (12-14). HPV is more commonly detected in oral condyloma acuminatum, but there is considerable overlap in the histopathological features of condyloma and papilloma of the oral mucosa (15,16). There is a strong association between HPV and dysplastic, exophytic oral lesions in immunosuppressed patients (2,17,18). In contrast, the prevalence of HPV in dysplastic and malignant oral verrucous or papillary lesions in immunocompetent patients is much lower (19-21).

This study examined a group of atypical and malignant papillary lesions for low and high risk HPV and correlated the detection of HPV with clinical and pathological features, with the aim of improving our understanding of these uncommon oral lesions.

4.3 Materials and Methods

4.3.1 Tissue Samples

This was a retrospective study of biopsy specimens of oral mucosal lesions submitted to the Oral Pathology Diagnostic Service (OPDS) at the Faculty of Dentistry, University of Toronto, by general and specialist dentists in the province of Ontario. This study was approved by the Research Ethics Boards of the University of Toronto (protocol #26571) and the University Health Network (protocol #12-5269-TE). A search of the OPDS files from 2005 to 2011 was conducted using the keyword “papillary.” A total of 28 atypical or dysplastic papillary lesions and 14 malignant papillary lesions were identified. For comparison with the atypical and malignant papillary lesions, 24 cases of squamous papilloma accessioned during the same period were also collected for study. Lesions diagnosed as inflammatory papillary hyperplasia of the
palate were excluded from the study, because this is well recognized as a reactive mucosal condition. No patients included in the study had a history of immunosuppression. Fourteen cases of normal oral epithelium from specimens diagnosed as fibroepithelial polyp or amalgam tattoo, from a variety of oral sites, were used as normal controls.

Clinical follow-up information on cases of APL and MPL was obtained from the clinician who submitted the biopsy. Some of the patients with persistent lesion after biopsy were referred to Head and Neck Surgery at the University Health Network (UHN) for further treatment. Follow-up information for these patients was obtained by chart review at UHN.

4.3.2 In situ DNA hybridization for High and Low Risk HPV

In situ hybridization (ISH) for HPV DNA was performed on 4 µm thick sections of formalin-fixed, paraffin-embedded (FFPE) biopsy specimens, using the Inform HPV II Family 6 probe (B) for low risk HPV and the Inform HPV III Family 16 probe (B) for high risk HPV on a Ventana Benchmark automated slide stainer according to the manufacturer’s instructions (Ventana Medical Systems, Inc., Tucson, AZ, USA), as previously described (22). A case of condyloma acuminatum was used as the positive control for the Family 6 probe and normal oral epithelium was used as the negative staining control. High risk HPV 3-in-1 control slides supplied by the manufacturer were used, including CaSki cells (HPV16 positive, 200-600 copies per cell), HeLa cells (HPV18 positive, 10 - 50 copies per cell) and T24 cells (HPV negative). In addition, a case of HPV16 positive oropharyngeal carcinoma was used as positive control for the Family 16 probe. ISH for HPV DNA was scored as positive when blue reaction product was seen in the nucleus of lesional cells, in either a homogeneous or punctate pattern. Staining that was not localized to the nucleus of epithelial cells was interpreted as background staining. All cases were scored by two observers (C.M. and G.B.) following the criteria above.

4.3.3 Immunohistochemical Staining

Immunohistochemical (IHC) staining for p16 protein was performed on 4µm sections of FFPE tissue using a monoclonal antibody to p16 (CINtec, Roche mtm laboratories, Westborough, MA, USA), and a Ventana Benchmark automated slide stainer, according to the manufacturer’s instructions (Ventana Medical Systems, Inc., Tucson, AZ, USA), as previously described (22). A case of HPV-positive oropharyngeal carcinoma was used as the positive staining control. Cases
of normal oral epithelium were used as negative staining controls. Staining for p16 protein was seen in both nucleus and cytoplasm of epithelial cells, in agreement with previous reports (23,24). The grading of p16 staining was in accordance with a previously described protocol (22). Briefly, p16 staining was scored as positive when there was strong, diffuse staining of lesional epithelium. Staining was scored as equivocal when there was patchy staining with unstained epithelial cells interspersed among cells with nuclear and cytoplasmic staining. Cases with no staining or staining of scattered, single cells within the lesion were scored as negative. All cases were scored by two observers (C.M. and G.B.).

Immunohistochemical staining for Ki-67 antigen was performed with the monoclonal antibody MIB-1 (Dako Canada, Burlington, Ontario). Normal oral epithelium was used as the control, where a subset of cells in the basal and parabasal layers showed staining of the nucleus. Cases were scored in accordance with a modification of a previously described protocol (22). Staining of basal and parabasal epithelial nuclei was scored as grade 1. Staining of nuclei in suprabasilar layers in addition to basal and parabasal layers was scored as grade 2. This modification of the previous protocol was necessary because the range of MIB-1 staining seen in the papillary lesions was less than that seen in oral epithelial dysplasia in the previous study (22).

4.3.4 Statistical Analysis

Descriptive statistics were provided as median and range for continuous variables and frequencies and proportions for categorical variables.

The utility of immunohistochemical staining for MIB-1 and p16 as markers for the presence of low risk HPV was examined by a test for agreement and Kappa coefficient was calculated.

The association between HPV infection status and clinical variables was analyzed in contingency tables, using the Fisher’s exact test and the Wilcoxon rank sum test. Two-sided tests were applied. Results were considered significant if the P value was <0.05. All the statistical analyses were performed with SAS 9.2 (SAS Institute, Cary, NC, USA).
4.4 Results

4.4.1 Clinical and Pathologic Findings

Twenty-eight atypical papillary lesions and 14 malignant papillary lesions were included in this study. Twenty-four papillomas were also studied for comparison. The clinical characteristics for the papillomas, atypical papillary lesions (APLs) and malignant papillary lesions (MPLs) are listed in Table 4.1. The median age for the APL group was 60 years, while the median age for the MPL group was older at 76 years. Both groups showed a similar age range. The median age for the papilloma group was 45. The most commonly affected site for all groups of papillary lesions was the gingiva. Eight of 24 squamous papillomas, 9 of 28 APLs, and 7 of 14 MPLs were removed from the gingiva. The soft palate was a relatively frequent site of involvement for papillomas with 4 cases, but was uncommon for APLs with only one case occurring at this site. None of the MPLs were seen in the soft palate. The size of the lesions was determined from the clinical description in cases of incisional biopsy, or the gross description verified by on-slide measurement in cases of excisional biopsy. The papillomas ranged from 0.25 to 1.0cm, whereas APLs ranged from 0.3cm to 7.6cm, and MPLs ranged from 1 cm to greater than 5cm.

All 24 cases of squamous papilloma showed the classical features of this lesion, consisting of a narrow, branching core of fibrovascular connective tissue covered by stratified squamous epithelium with a normal maturation pattern and no evidence of dysplasia. Some of the papillomas showed surface keratinization, depending on the intraoral site (1) (Figure 4.1 A, B).

All except one case of the atypical papillary lesions were distinguished from papillomas by histologic findings. The exception was a floor of mouth lesion described clinically as atypical because of the irregular shape and relatively large size. The other 27 APLs were exophytic growths with papillary projections that were covered by dysplastic epithelium, with no evidence of invasion into the connective tissue stroma. The rete ridges were wide and elongated, with epithelial dysplasia seen as increased nuclear/cytoplasmic ratio, nuclear hyperchromatism and pleomorphism predominantly affecting the deep half of the epithelium (Figure 4.1 C-F).
Table 4.1 Clinical features of papillary epithelial lesions

<table>
<thead>
<tr>
<th></th>
<th>Papillomas</th>
<th>Atypical Papillary Lesions</th>
<th>Malignant Papillary Lesions</th>
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<tbody>
<tr>
<td>Total</td>
<td>24</td>
<td>28</td>
<td>14</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (range)</td>
<td>45 (7-81)</td>
<td>60 (32-95)</td>
<td>76 (46-93)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>13</td>
<td>17</td>
<td>6</td>
</tr>
<tr>
<td>Female</td>
<td>11</td>
<td>11</td>
<td>8</td>
</tr>
<tr>
<td>Smoking history¹</td>
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<td></td>
</tr>
<tr>
<td>Non-smoker</td>
<td>n.d.</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Smoker</td>
<td>n.d.</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Site</td>
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<td></td>
<td></td>
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<tr>
<td>Gingiva</td>
<td>33.3% (8/24)</td>
<td>32.1% (9/28)</td>
<td>50% (7/14)</td>
</tr>
<tr>
<td>Floor of mouth</td>
<td>16.7% (4/24)</td>
<td>14.3% (4/28)</td>
<td>21.4% (3/14)</td>
</tr>
<tr>
<td>Tongue</td>
<td>12.5% (3/24)</td>
<td>21.4% (6/28)</td>
<td>7.1% (1/14)</td>
</tr>
<tr>
<td>Hard palate, palate NOS</td>
<td>4.2% (1/24)</td>
<td>3.6% (1/28)</td>
<td>7.1% (1/14)</td>
</tr>
<tr>
<td>Soft palate</td>
<td>16.7% (4/24)</td>
<td>3.6% (1/28)</td>
<td>0%</td>
</tr>
<tr>
<td>Lip</td>
<td>8.3% (2/24)</td>
<td>14.3% (4/28)</td>
<td>0%</td>
</tr>
<tr>
<td>Buccal mucosa</td>
<td>8.3% (2/24)</td>
<td>10.7% (3/28)</td>
<td>14.3% (2/14)</td>
</tr>
</tbody>
</table>

¹ Smoking history was not determined for papilloma cases, unknown in 12 APL cases and 5 MPL cases
Figure 4.1. Hematoxylin and eosin stained sections- A, squamous papilloma (x1). B, Higher magnification of same lesion shown in A (x10). C, HPV 6/11 positive atypical papillary lesion (x1). D, Higher magnification of same lesion shown in C (x10). E, HPV negative atypical papillary lesion (x 0.8). F, Higher magnification of same lesion shown in E (x10). G, Malignant papillary lesion (x 0.6). H, Higher magnification of the same lesion shown in G (x10).
The malignant papillary lesions were similar to the atypical papillary lesions, presenting as exophytic papillary lesions covered by epithelium with dysplasia that involved the deep half of the epithelial thickness or less commonly, the entire thickness. In contrast to the atypical lesions, there was invasion into connective tissue (Figure 4.1G,H).

We examined all cases for the presence of koilocytosis, seen as groups of upper spinous cells with an irregularly shaped (raisin-like) nucleus and clear cytoplasm. These histologic features have been shown to correlate with the presence of HPV in the epithelial cells (1). Koilocytosis was present in 50% of papillomas (12/24), 43% of APLs (12/28) and 36% of MPLs (5/14).

### 4.4.2 Immunohistochemical Staining for p16 Protein

Immunohistochemical staining for p16 was used as a sensitive marker for high risk HPV infection, according to previous reports that strong, diffuse staining of lesional epithelium correlated with detection of high risk HPV by ISH (22,24,25). Strong, diffuse staining for p16 was not observed in any of our cases in this study. A patchy pattern of p16 staining (Figure 4.2), in which groups of lesional cells with nuclear and cytoplasmic staining were interspersed with cells with no staining, was found in 20% of papillomas (2/10), 57.1% of APLs (16/28), and 28.6% of MPLs (4/14) (Table 4.2).
Figure 4.2. Patchy staining for p16. A, HPV negative malignant papillary lesion (x25). B, Higher magnification of the same lesion shown in A (x200). C, HPV 6/11 positive atypical papillary lesion (x25). D, Higher magnification of the same lesion shown in C (x200).
### Table 4.2 Results of analyses for p16, Low risk and High risk HPV and MIB-1

<table>
<thead>
<tr>
<th></th>
<th>p16 patchy staining</th>
<th>Low risk HPV positive</th>
<th>High risk HPV positive</th>
<th>MIB-1 basal/parabasal</th>
<th>MIB-1 suprabasal</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Papillomas</strong> (n=24)</td>
<td>20.0% (2/10)&lt;sup&gt;1&lt;/sup&gt;</td>
<td>4.2% (1/24)</td>
<td>n.d.&lt;sup&gt;2&lt;/sup&gt;</td>
<td>90% (9/10)&lt;sup&gt;1&lt;/sup&gt;</td>
<td>10% (1/10)&lt;sup&gt;1&lt;/sup&gt;</td>
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<td></td>
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<tr>
<td><strong>Atypical Papillary Lesions</strong> (n=28)</td>
<td>57.1% (16/28)</td>
<td>32.1% (9/28)</td>
<td>0% (0/16)&lt;sup&gt;3&lt;/sup&gt;</td>
<td>60.7% (17/28)</td>
<td>39.3% (11/28)</td>
</tr>
<tr>
<td></td>
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<td></td>
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<td></td>
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</tr>
<tr>
<td><strong>Malignant Papillary Lesions</strong> (n=14)</td>
<td>28.6% (4/14)</td>
<td>0% (0/14)</td>
<td>0% (0/4)&lt;sup&gt;3&lt;/sup&gt;</td>
<td>93% (13/14)</td>
<td>7% (1/14)</td>
</tr>
</tbody>
</table>

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1. 10 of 24 papillomas were stained for p16 and MIB-1
2. not done
3. ISH for High risk HPV was done for atypical and malignant papillary lesions with p16 staining, not done for lesions with no p16 staining
4.4.3 In situ Hybridization for HPV DNA

All cases included in the study were investigated for low risk HPV DNA with ISH. Ten of 66 cases, including 9 cases of APL and 1 papilloma, demonstrated evidence of low risk HPV DNA. Evidence of low risk HPV was not found in any of the MPLs (Table 4.2). All positive cases showed a homogeneous hybridization signal within the nuclei of lesional cells (Figure 4.3), consistent with the presence of episomal viral DNA. A punctate pattern of hybridization, suggestive of integrated viral DNA, was not seen in any of the positive cases. Groups of labelled nuclei were restricted to the superficial aspect of the lesional epithelium, and were not present in the basal or parabasal layers of the papillary proliferation or in the adjacent unaffected mucosa.

To investigate whether the patchy p16 staining seen in a subset of APLs and MPLs was indicative of high risk HPV infection, all cases showing patchy p16 staining were examined for high risk HPV DNA (Table 4.2). None of the lesions showed evidence of high risk HPV DNA by ISH, indicating that the patchy p16 staining pattern was not associated with the presence of high risk HPV (22).
Figure 4.3. A and B, in situ hybridization for HPV 6/11 showing homogeneous signal in lesional nuclei (x50 and x200). C and D, Another case showing a similar signal pattern (x50 and x200).
4.4.4  **Immunohistochemical Staining for MIB-1**

Immunohistochemical staining for MIB-1 was used to indicate the distribution of proliferative cells within the epithelium. In the stratified squamous epithelium adjacent to the papillary lesions, proliferative cells were restricted to the basal and parabasal layers. All 10 cases of papillary lesions that were positive for low risk HPV by ISH showed MIB-1 staining in suprabasal layers (Table 4.2). The MIB-1 positive nuclei were scattered throughout the epithelial layers rather than localized to the basal and parabasal layers (Figure 4.4 E,F). This pattern of suprabasal MIB-1 staining was notably different from the pattern that was previously described for high risk HPV-positive epithelial dysplasia, where the majority of nuclei in basal, parabasal and suprabasal layers were positive, indicating a high proliferative index (22). MIB-1 staining of suprabasal nuclei was also seen in 2 cases of APL and 1 case of MPL that were negative for low risk HPV. The other papillary lesions (9 of 10 papillomas, 17 of 28 APL, and 13 of 14 MPL) showed MIB-1 staining that was restricted to basal and parabasal layers (Figure 4.4 A-D). The basal and parabasal distribution of proliferative cells in 13 of 14 MPL cases was consistent with the predominantly well differentiated histomorphology of these lesions.

4.4.5  **Immunohistochemical markers for Presence of Low Risk HPV**

The results of MIB-1 and p16 staining were compared with ISH for low risk HPV, to determine if one or both immunohistochemical assays could be used as an indirect test for low risk HPV in oral papillary lesions. For the 52 cases of papilloma, APL and MPL where all 3 assays were done, the agreement of MIB-1 staining to ISH for low risk HPV was assessed, and similarly for p16 staining to ISH for low risk HPV (Table 4.3). There was strong agreement between suprabasal MIB-1 staining and detection of low risk HPV (Kappa coefficient=0.83). There was less agreement between patchy p16 staining and detection of low risk HPV (Kappa coefficient=0.41) because 13 of 42 cases that were HPV negative also showed patchy p16 staining (Table 4.3).
Figure 4.4. MIB-1 Immunohistochemistry. A, Staining of basal and parabasal nuclei in a squamous papilloma (x12.5). B, Case A at higher magnification (x50). C, Staining of basal and parabasal nuclei in a HPV negative atypical papillary lesion (x12.5). D, Case C at higher magnification (x50). E, Staining of suprabasal nuclei in a HPV 6/11 positive atypical papillary lesion (x12.5). F, Case E at higher magnification (x50).
Table 4.3 Test for agreement of MIB-1 to low risk HPV and p16 to low risk HPV

<table>
<thead>
<tr>
<th></th>
<th>HPV negative</th>
<th>HPV positive</th>
<th>Kappa coefficient (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIB-1 staining</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal and parabasal</td>
<td>39</td>
<td>0</td>
<td>0.83 (0.65 – 1)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Suprabasal</td>
<td>3</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p16 staining</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No staining</td>
<td>29</td>
<td>1</td>
<td>0.41 (0.18 – 0.63)</td>
<td>0.0007</td>
</tr>
<tr>
<td>Patchy</td>
<td>13</td>
<td>9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4.4.6 Clinical features and outcome of APL cases

The clinical parameters of low risk HPV positive and negative cases of APL were compared (Table 4.4). There was a predominance of men among the HPV positive cases (M:F ratio of 8:1) that was not present in the HPV negative cases (p=0.05, Fisher’s exact test). The HPV positive APL group showed a younger median age and a higher proportion of smokers, but the differences were not statistically significant.

Clinical follow-up information was obtained for 17 of the 28 patients with APL. For 10 patients (4 HPV positive and 6 HPV negative), the lesion was excised and did not recur after a follow-up period that varied from 4 to 46 months with a median of 16.5 months. Within the HPV positive group, one patient had 5 recurrences at the same site over 50 months. Another patient developed a recurrent 2 mm lesion at 9 month follow-up, which was excised with no further recurrence. None of the HPV positive cases progressed to carcinoma. Within the HPV negative group, one patient had a recurrent lesion at 21 month follow-up, which was excised. For another patient in this group, an 86 year-old woman, the submitted specimen was an incisional biopsy of a large, papillary mucosal lesion. No definitive treatment was provided because of poor health status. In three patients in the HPV negative group, there were recurrent or persistent lesions that progressed to carcinoma (Table 4.4).
**Table 4.4** Clinical parameters and follow-up information for APL cases

<table>
<thead>
<tr>
<th></th>
<th>HPV 6 positive (n=9)</th>
<th>HPV 6 negative (n=19)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (range)</td>
<td>53 (32-82)</td>
<td>76 (46-95)</td>
<td>0.10</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>8</td>
<td>9</td>
<td>0.05</td>
</tr>
<tr>
<td>Female</td>
<td>1</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td><strong>Smoking Status</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoker</td>
<td>5</td>
<td>4</td>
<td>0.36</td>
</tr>
<tr>
<td>Non-Smoker</td>
<td>2</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td><strong>Follow-up</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No recurrence</td>
<td>4</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Recurred/persisted</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>progressed</td>
<td>0</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

1 Wilcoxon test
2 Fisher’s exact test
3 Unknown in 2 HPV positive and 10 HPV negative cases
4 Unknown in 3 HPV positive and 8 HPV negative cases
4.4.7 Clinical Outcome of MPL cases

Clinical follow-up was available for 10 of the 14 patients with MPL. One patient died of unrelated causes 54 months following biopsy of the oral lesion. Seven patients were alive without disease at last follow-up that varied from 34 months to 71 months (median 43 months). One patient with a buccal mucosal lesion was treated with primary radiation therapy; recurrence at 8 months was treated with surgery. She then developed a new lesion at 17 months and was treated with palliative care only. Another patient, an 85 year-old woman, did not undergo definitive treatment because of poor health and was alive with disease at 5 month follow-up. None of the patients with follow-up has died of the oral papillary malignant disease.

4.5 Discussion

The association between HPV and the spectrum of oral papillary epithelial proliferations has not been clearly established. The variation in findings reported in the literature may be due to case selection, inclusion of immunocompromised patients, and use of different techniques for HPV detection. We used a combination of clinical data, histopathological examination and ISH for HPV to demonstrate an association between low risk HPV and a subset of atypical papillary lesions which ran a benign course, although the duration of follow-up was relatively short. None of the lesions had detectable high risk HPV. Some of the HPV-negative, atypical papillary lesions showed persistent growth and progression to carcinoma. HPV was not detected in any of the malignant papillary lesions. Thus, our findings suggest that potentially malignant and malignant papillary oral lesions were not associated with low or high risk HPV. Ten of the 66 oral papillary lesions in our study showed low risk HPV by ISH. One of 24 papillomas was HPV positive and this was the largest lesion within the papilloma group. HPV was detected in 9 of 28 APLs, which are papillary lesions distinguished from simple squamous papillomas by a combination of larger size, irregular shape and epithelial atypia or dysplasia seen as nuclear hyperchromatism and increased nuclear:cytoplasmic ratio in the deep half of the epithelium. All cases that were low risk HPV positive showed scattered MIB-1 positive nuclei in the suprabasal epithelium, in contrast to the localization of MIB-1 staining to basal and parabasal epithelial cells that constitute the normal proliferative compartment (Table 4.2). These findings
suggest that infection by low risk HPV results in aberrant epithelial proliferation and differentiation. Follow-up data was available for 6 of 9 low risk HPV positive APLs. Two patients had recurrent lesions at the same site that were successfully excised. None of the low risk HPV positive lesions progressed to carcinoma.

High risk HPV was not detected in any of the papillary epithelial lesions. Previous studies have shown the association of high risk HPV with a subset of severe epithelial dysplasia and oral carcinoma. The HPV associated severe epithelial dysplasias have a distinctive histologic appearance of full thickness loss of normal maturation and evidence of individual cell death throughout the epithelium (22,26). These lesions also show diffuse, strong p16 immunostaining, due to inactivation of pRb by the E7 oncoprotein of high risk HPV. In contrast, the low risk HPV positive papillary lesions in this study showed patchy p16 staining or no staining. Thus, low risk and high risk groups of HPV can both infect oral squamous epithelium but the pathology of the resulting lesions is different. Recent studies have suggested that these differences may reflect the ability of the E6 and E7 proteins of low risk versus high risk HPV to interact with cell cycle regulators, particularly pRb and p53 (27).

The utility of MIB-1 and p16 immunostaining as an indirect assay for low risk HPV was examined. There was strong agreement between MIB-1 staining of suprabasal epithelial cells and detection of low risk HPV. There was only moderate agreement between patchy p16 staining and presence of low risk HPV (Table 4.3). Further studies of oral papillary epithelial lesions are needed to confirm that loss of localization of MIB-1 staining to the basal and parabasal layers is a good screening test for low risk HPV infection.

Some of the APLs exhibited wide, elongated rete ridges and koilocytosis in the upper spinous layers, which raised the possibility that these were lesions of condyloma acuminatum. However, none of the APLs were associated with a clinical history of contact with genital condyloma. Furthermore, we could not demonstrate a correlation between the histologic finding of koilocytosis and the detection of low risk HPV by ISH. The inconsistent presence of koilocytes in oral lesions of condyloma makes it difficult to distinguish between oral papilloma and condyloma (1,15). The routes of transmission of HPV to mucosal epithelium have not been fully established. To avoid the implication of oral-genital contact, it is preferable to use the diagnosis of HPV6/11 positive papillary epithelial lesion for the ISH positive cases reported here.
Nineteen of 28 APLs were negative for low and high risk HPV. These cases appeared to differ clinically from the 9 cases of low risk HPV positive APLs, although the differences did not reach statistical significance of $p <0.05$ (Table 4.4). Most notably, the HPV negative cases did not share the male predominance of the HPV positive cases. Among the 11 cases with follow-up, there were 3 patients whose APL represented an incisional biopsy of a large papillary lesion. One patient did not undergo further surgery because of poor health. The other two patients were subsequently diagnosed with verrucous carcinoma and well differentiated squamous cell carcinoma, respectively. Two patients had a recurrent APL of the gingiva, and the clinical history was consistent with proliferative verrucous leukoplakia. One of these patients progressed to well differentiated squamous cell carcinoma at 62 months following the initial biopsy of APL. Thus, at least some of the HPV negative APLs represented potentially malignant lesions with a papillary growth pattern.

The MPLs presented clinically as extensive lesions, in some cases involving more than one anatomical site. The histologic findings were similar to APLs in that the lesion consisted of papillary projections covered by dysplastic epithelium, but MPLs also showed invasion of the connective tissue (Figure 4.1 G, H). In this study, we excluded cases of squamous cell carcinoma with limited areas of papillary surface growth, or a rough surface due to multiple invaginations of the malignant epithelium. Information on smoking was available in 9 of the 14 patients with MPLs. Eight of the 9 patients were non-smokers and one patient was a former light smoker (1-2 cigs/day). Seven of 14 MPLs were found on the gingiva (Table 4.1). Among the 10 patients with follow-up available, none has died of disease. MPLs appear to be low grade squamous cell carcinomas with a prolonged phase of exophytic, superficial growth. Histologic diagnosis of these lesions as carcinoma may be problematic because multiple biopsies may be needed to demonstrate the presence of stromal invasion. We did not detect HPV in MPLs, which agrees with recent reports of a low prevalence of HPV in oral cavity carcinomas, including carcinomas with a verrucous or papillary architecture (21,28,29).

4.6 Conclusion

Our study of HPV in a spectrum of oral papillary lesions suggests that simple squamous papillomas are rarely HPV positive. Instead, low risk HPV (HPV 6 and 11) infection is found in benign papillary proliferations with virally induced atypia. These low risk HPV positive APLs
may be distinguished from APLs that are potentially malignant lesions because the latter group is not associated with HPV. MPLs are not associated with low or high risk HPV. The number of cases in this study is small, and our results should be confirmed in further studies of biologically significant HPV infection in oral papillary lesions.

4.7 Acknowledgements

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Grant support - Dental Research Institute, Faculty of Dentistry

4.8 References


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Chapter 5. Discussion

HPV infections are ubiquitous. It is currently estimated that 79 million Americans are infected with HPV, and that 50% of sexually active adults will become infected with HPV in their lifetime (51,190). HPV related oropharyngeal cancers are a growing public health concern. Between 1988 and 2004, it is estimated that the incidence of HPV positive oropharyngeal carcinoma increased by 225%, while the incidence of HPV negative oropharyngeal carcinoma declined by 50% over the same time period (52,119). The investigation of the association of HPV with oral lesions is a topical area of research because of the proximity of the oral mucosa to the oropharyngeal mucosa. The study of oral lesions also has epidemiologic importance, given that oral infections are quite common, currently estimated at 7% (52), and that oral infection with HR HPV is thought to increase the risk of developing a HPV positive HNSCC (52,124). To date, however, significant gaps remain in our knowledge of the spectrum of HPV associated oral lesions, especially with respect to HPV associated premalignant lesions (51). In fact, much of the published data have led to confusion about the association of HPV with oral cancer and oral lesions in general.

Current data from the HPV literature supports an overarching theme: not all HPV infection results in disease. The cervical cancer literature shows that most cervical HPV infections clear within 2 years, regardless of whether they induce local cytologic changes or not (33,191). Persistence of viral infection is known to be associated with increased risk of cervical precancer, though the exact mechanisms that determine which infections persist and which infections are cleared are not well understood (31,33). When compared to cervical infections, significantly less is known about the natural history of oral HPV infections, and preliminary evidence suggests that the natural history may be different (51,137). Despite the lack of knowledge about the evolution of oral HPV infections, the comparative infrequency of HPV related oropharyngeal cancers (2.6 per 100,000 population) (119) relative to the frequency of oral HPV infection suggests that most oral infections must be transient, and therefore clinically insignificant.

The failure to recognize the importance of distinguishing between transient versus persistent HPV infection underlies much of the controversy about the prevalence of HPV in oral lesions.
Though exquisitely sensitive, detection of HPV by conventional PCR-based methods does not allow for distinction between clinically relevant (persistent, transcriptionally active), and transient infections (73). Merely demonstrating the presence of viral DNA within a tissue sample does not provide information about whether the infection is biologically active or not. Therein lies the benefit in using ISH based methods over PCR for viral DNA detection. Although a lower sensitivity increases the potential for false negatives, ISH allows for demonstration of viral DNA within the nuclei of lesional tissue (77). Additionally, the pattern of the ISH signal, punctate versus homogeneous, provides information on the physical state of the virus, whether integrated within the host genome or episomal (56,82,83). Viral integration into the host genome is thought to be important in the progression to cancer (14).

The ability of high risk HPV to cause malignant transformation is dependent on the expression of viral oncoproteins E6 and E7 (14,17). Thus, viral E6/E7 mRNA detection by reverse transcriptase PCR (RT-PCR) is considered to be the gold standard for the detection of biologically relevant HPV infection (71,73,74). However, RT-PCR is technically challenging, requires more tissue, and therefore may not be practical in a clinical setting (56,71,73,74,80,81). p16 immunohistochemistry is widely accepted as a sensitive, surrogate marker for transcriptionally active HR HPV infection (74). p16 overexpression results from inactivation of pRb by viral protein E7 and disruption of negative feedback regulation between pRb and p16 (9,192,193). The use of p16 immunohistochemistry and ISH, therefore, combines the high sensitivity of p16 with the high specificity of ISH, and is practical, cost-effective and can be routinely applied to formalin-fixed, paraffin embedded tissues (73, 82). We studied the association of HR HPV with oral lesions using this well-supported algorithm to detect transcriptionally active viral infection.

Case selection and population differences should also be considered when evaluating reports of the role of HPV in oral potentially malignant lesions. Previous studies have often examined a mixed group of oral potentially malignant lesions, ranging from hyperkeratosis, to lichen planus to dysplasia (4,143,147). As the etiopathogenesis and malignant potential of many of these oral lesions differ significantly, a research strategy that investigates the association of HPV within a defined subset of lesions is more likely to be informative. We excluded lesions from immunocompromised individuals from our study, as it is known that immunosuppression is a
significant risk factor for viral persistence and therefore viral-related oncogenesis (28,31,32). Furthermore, HPV infections are more common in HIV infected individuals, and HPV subtypes are more variable than those seen in immunocompetent persons (28,33). Though important, data from the study of immunocompromised individuals are not readily generalized to the population at large, and it is therefore important to control for this potential confounding factor.

One of the most interesting emerging themes from the HPV literature is that the biologic behavior of HPV seems to depend on the site of infection. The clinical and histopathologic manifestations of HPV vary considerably depending on the type of epithelium infected. In the uterine cervix, infection with HR HPV is a necessary, but not sufficient cause for the development of cervical cancer (28,32,194). Furthermore, 99% of cervical cancers harbour oncogenic HPV types (195). Cervical cancers preferentially arise from the cervical transformation zone, where there is a transition between stratified squamous epithelium and glandular epithelium (33). It is thought that the transition areas are important for the development of HPV induced cancers. Similar to cervical cancers, oropharyngeal and anal cancers are also known to arise at epithelial transition zones (33,196). Cervical pre-cancer is also associated with HPV infection. At the low grade end of the spectrum, low grade squamous intraepithelial lesions (LSIL) demonstrate infection with a broad spectrum of HPV types, both high and low risk (9). In a small number of cases, HPV infection persists and there is progression to a high grade lesion (HSIL) and, if untreated, subsequently SCC or adenocarcinoma of the cervix (28). As the severity of the cervical lesion increases from LSIL to HSIL, the prevalence of HPV infection correspondingly increases, and the spectrum of HPV types causing the infection changes (9). For example, HPV type 16 is 2.5 times more common in HSIL versus LSIL (9). LR HPV types 6 and 11 are also known to cause benign warty lesions of the genital tract, such as condyloma accuminatum.

HPV infection in the oral cavity is associated with a similar spectrum of lesions as the uterine cervix, with several important differences. Although oral HPV infection is common, it is decidedly less frequent than cervical HPV infection (52,69,70). Unlike the cervix, the incidence of HPV related oral cancer is low, with current estimates around 6% of all oral cancers (79). Our results have shown, in accordance with other studies (197,198), that HR HPV is associated with a subset of high grade oral epithelial dysplasia. HPV was present in only 7 of 40 high grade
dysplasias in our study, suggesting that this is an infrequent event. In contrast to cervical lesions (33,199), we did not find that LR HPV was associated with any oral dysplasias, irrespective of grade. Therefore, in contrast to cervical HPV infections, HPV infection is not a common or necessary cause of oral cancer or pre-cancer.

Similar to the anogenital tract, LR HPV subtypes are associated with oral papillary lesions. Nine of 28 atypical papillary lesions in our study were found to contain LR HPV DNA. The atypical papillary lesions are differentiated from conventional squamous papillomas because of clinical and/or histological atypia. Despite the presence of atypical features, all of the HPV positive cases behaved in a benign fashion. Our data suggest that HPV is not associated with development of malignant oral papillary lesions. Conventional squamous papillomas were studied for comparison with APLs and MPLs. It was somewhat surprising, however, that only 1 papilloma of 24 demonstrated LR HPV positivity. This frequency is lower than that reported in other investigations of squamous papillomas that used ISH based methods (109,110,129). Eversole et al. (109) found that HPV DNA was present in 35% of papillomas studied (7/20), while Fregonesi (110) found 14% (2/14) of squamous papillomas were positive for LR HPV. Possible explanations for the low level of LR HPV found in squamous papillomas by ISH based methods include viral copy number below the detection level of the ISH assay, loss of HPV viral activity over time, and a pathogenesis unrelated to HPV (109). The results of our current study suggest that many of the small, conventional squamous papillomas are not related to LR HPV infection, however further investigation is warranted.

HPV positive cancers of the oropharynx also demonstrate distinctive, site specific features and behaviour. HR HPV positive oropharyngeal squamous cell carcinomas are not known to have a precursor lesion (82), which is dissimilar to both the oral cavity and the cervix. The appearance of the carcinomas in the oropharynx is thought to recapitulate the reticulated epithelium of the tonsillar crypt, assuming a basaloid, non-keratinizing appearance. In the oral cavity, SCC harboring oncogenic HPV types is uncommon, and the histopathology of the lesions is variable and includes both keratinizing and non-keratinizing squamous cell carcinomas (79). Oropharyngeal cancers, like cervical cancers, have a strong site predilection, with the majority of cases affecting the palatine tonsils and base of tongue. Oral HPV positive SCC, on the other hand, has a similar site distribution as HPV negative SCC (79). Although the site predilection of
HR HPV positive dysplasias in our study did not reach statistical significance, the majority of cases affected the floor of the mouth/ventral tongue, suggesting that this may be a high risk site.

Though the sinonasal tract is not considered to be frequent site of HPV related cancers, recent research suggests that HPV may be a causative factor in up to 21% of cases of sinonasal carcinoma (200,201). As in other anatomic sites, it appears that at least some HPV related sinonasal cancers show distinctive histologic features. Bishop et al. (201) recently identified a subset of HPV positive sinonasal carcinomas with histologic similarity to adenoid cystic carcinoma of salivary glands. These tumours were often associated with surface dysplasia, supporting their mucosal, rather than salivary gland origin, and HPV 33 was the most common subtype identified.

The primary aim of our research was to further understanding of the spectrum of oral lesions associated with HPV. We have shown that the combination of ISH, p16 and MIB-1 in FFPE tissues is a reliable and practical approach for studying HPV in oral lesions. Although the use of ISH and p16 is a commonly used algorithm, the use of MIB-1 is not common in the evaluation of HPV-related lesions of the head and neck. In the uterine cervix, cervical intraepithelial neoplasia (CIN) cases infected with HR HPV show a higher proliferation index than those infected with LR HPV subtypes (202). Furthermore, increasing MIB-1 positivity is associated with increasing severity of dysplasia (99). Our results demonstrate that oral lesions caused by HR and LR HPV have distinctive MIB labelling patterns. HR HPV positive high grade dysplasias show a very high MIB labelling index, involving at least 2/3 of the epithelial thickness, which suggests that a high proportion of cells are actively dividing. In normal stratified squamous epithelium, proliferating cells are limited to the basal and parabasal layers of the squamous epithelium, reflecting the normal asymmetric division of somatic stem cells (203). HR HPV is thought to disrupt both the pRb and p53 pathways, leading to aberrant cell proliferation. Therefore a higher proliferative index throughout the thickness of the epithelium is in keeping with our understanding of the biologic activities of HR HPV. In LR HPV positive oral papillary lesions, MIB-1 positive nuclei were present throughout the suprabasal epithelium, and the distribution of positive nuclei had a distinctive, scattered, histopathologic pattern. In the perilesional epithelium, the proliferating cells show a normal distribution, limited to the basal and parabasal layers. To the best of our knowledge, this pattern of MIB labelling has not been previously
reported in association with LR HPV infection. The agreement between low risk HPV and suprabasal MIB staining was highly statistically significant (p<0.0001), suggesting that MIB-1 may be a useful adjunct for characterizing LR HPV positive lesions.

As previously discussed, p16 is widely accepted as a sensitive surrogate marker for transcriptionally active HR HPV infection. In studies of the oropharynx and other areas of the UADT, p16 positivity is largely determined based on the percentage of cells stained within the sample (150,204). Although a precise cut-off value has not been established, nuclear and cytoplasmic staining present in >50% of cells is an accepted definition of positivity (150). Our results, however, support the concept that the staining pattern, rather than the percentage of stained cells, is a more reliable predictor of HPV involvement. A strong, diffuse staining pattern involving the entire thickness of the epithelium is seen in all cases of HR HPV positive oral dysplasia. This association has been documented in other studies (151,198). Despite the strong association between diffuse p16 staining and HR HPV, p16 overexpression is not completely specific for HR HPV infection. In our series of epithelial dysplasias, four of eleven p16 positive cases did not show evidence of HPV DNA by ISH. The mechanism p16 overexpression in these cases is not known, however infection by other HPV subtypes or non-HPV related disturbances to the pRb pathway are plausible explanations (73). Lingen et al. (79) reported that up to 38% of HPV positive oral carcinomas in their study were attributed to HR HPV types other than HPV 16 including HPV 18, 31,33,35,39,45,51 and 52. In contrast to the strong, diffuse pattern of p16 staining, a patchy, mosaic-like pattern of p16 staining is non-specific. Although a patchy staining pattern was present in many of our LR HPV positive papillary lesions, it was also seen in one case of high grade dysplasia, and several cases of low grade dysplasia, squamous papilloma, APL and MPL that were negative for LR and HR HPV. Although p16 is currently not recommended for use as a stand-alone marker for assessing HR HPV infection, it is a reliable screening tool for HR HPV infection when appropriate criteria are used to evaluate the staining.

To determine the biologic potential of HPV associated oral lesions, we collected follow-up information on high grade epithelial dysplasia (Appendix A) and atypical papillary lesions, and compared HPV positive versus HPV negative cases in each group. In this retrospective study of biopsy cases, the duration of follow-up was relatively short, and follow-up was not available in all cases. Nevertheless, we observed differences in behaviour between HPV positive and
negative lesions. Among the 7 cases of HPV positive high grade dysplasia, one case was located adjacent to a squamous cell carcinoma which was also positive for HR HPV. None of the other HR HPV positive dysplasias progressed to carcinoma, and no lesional recurrences were reported (median follow-up 32 months, range 26-58 months). Six of the patients with HR HPV positive dysplasia were alive and free of disease at last follow-up, and one patient died of lung cancer at 37 months. In contrast, among the 28 cases of HPV negative high grade dysplasia with available follow-up, 12 cases were associated with carcinoma, including 8 cases where carcinoma was diagnosed 6 months or less after the diagnosis of dysplasia. The short time span in these cases suggests that the dysplasia was co-existent with carcinoma that was not sampled in the initial biopsy. Two patients had a recurrence of their carcinoma, and one patient developed a nodal metastasis 15 months after developing carcinoma. Two patients are known to have persistent white lesions, and four patients are known to have had recurrences of their dysplasia. Follow-up information was not available for 5 cases. With respect to risk factors, results suggest that the distribution of smoking was similar between the HPV positive and HPV negative groups. In the literature, outcome data for HR HPV positive oral dysplasias is extremely limited. Woo et al. (198) recently reported that one case of HR HPV positive dysplasia in their series progressed to carcinoma at 25 months, and one case presented with a concurrent carcinoma.

Follow-up data were available for 17 of the 28 APLs. The data suggest that APLs are a heterogeneous group of lesions. At one end of the spectrum, APLs behave in an indolent, benign manner with infrequent recurrence and no malignant progression. A small number of these indolent APLs are LR HPV positive. Conversely, some HPV-negative APLs are potentially malignant lesions that may progress to carcinoma. In our study, two patients that showed progression to carcinoma had a clinical history that was compatible with proliferative verrucous leukoplakia.

Previous studies have suggested that papillary squamous cell carcinomas may have a slightly improved prognosis compared with conventional SCC (161,165,169). Our follow-up data, while limited, show that none of the patients with an malignant papillary lesion has died of disease. We suspect that a primarily exophytic growth pattern may reflect a prolonged period of localized proliferation and destruction before invasion, and therefore these lesions may behave less aggressively than conventional SCC.
5.1 Study Limitations

Our study was limited by small numbers of cases, short duration of follow-up and lack of available follow-up for all cases. The rarity of the oral lesions that we investigated makes a prospective study design impractical. Therefore, while a retrospective case-series experimental design does not allow for determination of causality, it is practical for generating hypotheses and identifying areas of importance for future, larger scale and multi-centre studies.

5.2 Future Directions

Larger scale studies with follow-up information are needed in order to better understand the biologic behaviour of HR HPV positive dysplasias, and to assess whether differences exist between these lesions and conventional dysplasias. Further, although it appears that progression to carcinoma is a rare event, it is not presently known how carcinomas arising from HPV positive dysplasias behave. Although our results do not support evidence of a low grade variant of HR HPV positive dysplasia, further investigation is needed in order to definitively support this.

The mechanisms driving the overexpression of p16 in HPV-negative high grade dysplasias have not been elucidated thoroughly, and the clinical significance associated with the overexpression of p16 in these lesions is still unclear.

The prevalence of both atypical and malignant papillary lesions is low in the oral cavity. At present, follow-up data are very limited, and larger scale studies are needed. In particular, investigation of the use of LR HPV to help distinguish between potentially malignant atypical papillary lesions and atypical papillary lesions that demonstrate an indolent and benign course may provide information useful for clinical decision making.
Chapter 6. Conclusion

Human papillomavirus infection, high or low risk, is not a common finding in oral cavity lesions. Our data show that high risk HPV types are associated with a subset of high grade oral epithelial dysplasias with characteristic histopathologic features, which preferentially affect the floor of the mouth. High risk HPV positive dysplasias are diffusely p16 positive and show a distinctive increase in MIB-1 labelling, consistent with disruption of normal cell proliferation and maturation. Our data do not support an association of HPV with low grade oral epithelial dysplasia. The limited follow-up data that we obtained for the HPV positive high grade dysplasia group suggest that recurrence after excision and transformation to carcinoma are rare events.

Our findings indicate that oral papillary lesions are a heterogeneous group. Low risk HPV, but not high risk HPV, is associated with a small number of atypical papillary lesions that have a benign clinical course and infrequent recurrence. Low risk HPV produces a characteristic suprabasilar MIB-1 pattern by immunohistochemistry. The atypical histomorphology and abnormal pattern of MIB-1 labelling are interpreted to be a manifestation of viral infection. Among the HPV negative atypical papillary lesions, at least some are potentially malignant lesions. Low risk HPV may be helpful in identifying atypical papillary lesions that are unlikely to show potentially malignant behaviour. Malignant papillary lesions are large and locally destructive, but rarely result in death from disease. The pathogenesis of malignant papillary lesions is not associated with HPV.
References


(71) Snijders PJF, Heideman DAM, Meijer CJLM. Methods for HPV detection in exfoliated cell and tissue specimens. APMIS 2010;118(6-7):520-528.


Appendices

APPENDIX A. Clinical and follow-up information for high grade dysplasias.

<table>
<thead>
<tr>
<th>Case</th>
<th>Age</th>
<th>Sex</th>
<th>Site of primary tumour</th>
<th>HPV</th>
<th>History of smoking</th>
<th>Progression to carcinoma</th>
<th>Duration of follow-up</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>29</td>
<td>F</td>
<td>Floor of mouth</td>
<td>Pos</td>
<td>Yes</td>
<td>No</td>
<td>27 mos</td>
<td>Alive free of disease</td>
</tr>
<tr>
<td>2</td>
<td>55</td>
<td>F</td>
<td>Buccal mucosa</td>
<td>Neg</td>
<td>Yes</td>
<td>No</td>
<td>47 mos</td>
<td>Alive, white reticular lesions, multifocal, no dysplasia</td>
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<td>3</td>
<td>69</td>
<td>M</td>
<td>Floor of mouth</td>
<td>Pos</td>
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<td>Yes (concurrent)</td>
<td>58 mos</td>
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<tr>
<td>4</td>
<td>84</td>
<td>M</td>
<td>Lateral and ventral tongue</td>
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<td>No</td>
<td>Unknown</td>
<td>3 mos</td>
<td>Recurred as Moderate dysplasia</td>
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<td>5</td>
<td>76</td>
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<td>Yes (date unknown)</td>
<td>unknown</td>
<td>Progressed to SCC, date unknown</td>
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<td>6</td>
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<td>Yes (2mos)</td>
<td>55 mos</td>
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<td>Lower lip</td>
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<td>No</td>
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<td>Deceased lung cancer at 37 mos</td>
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<td>No</td>
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<td>Recurrence</td>
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<td>Sex</td>
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<td>Biopsy Status</td>
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mild dysplasia (2009); recurrence SCC (May 2012)
<table>
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<tr>
<th></th>
<th>Age</th>
<th>Sex</th>
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<th>Status 1</th>
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<td>Neg</td>
<td>Former</td>
<td>No</td>
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<tr>
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<td>Tongue, NOS</td>
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<tr>
<td>40</td>
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<td>Mandibular gingiva</td>
<td>Pos</td>
<td>Former</td>
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<td>26 mos</td>
<td>Alive, free of disease</td>
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