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Molecular characterization of human papillomavirus in head and neck tumors.

Sujita Khanal

MOLECULAR CHARACTERIZATION OF HUMAN PAPILLOMAVIRUS IN HEAD AND NECK TUMORS

By

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B.Sc., Purbanchal University, Nepal, 2007

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A Dissertation Approved on

July 15, 2016

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ABSTRACT

MOLECULAR CHARACTERIZATION OF HUMAN PAPILLOMAVIRUS IN HEAD AND NECK TUMORS

Sujita Khanal

July 15, 2016

Head and neck cancers (HNCs) are common causes of cancer-related deaths worldwide. An increasing incidence of subsets of HNCs is due to human papillomavirus (HPV). Other subsets are associated with tobacco and alcohol use. Determination of differential biomarkers and molecular mechanisms distinguishing HPV-associated and unassociated HNCs should improve diagnostic and therapeutic strategies. This study was designed to identify potential biomarkers for HPV detection, and to evaluate viral and host genetic and epigenetic mechanisms involved in HPV-associated HNCs.

Overexpression of a cellular protein, $p16^{INK4a}$, which is widely used as a surrogate marker for HPV-positive HNC, is non-specific because some HPV-negative cancers can overexpress $p16^{INK4a}$. I found “mitosoid cells” and “HPV E7 serology”, respectively, as histologic and serum biomarkers, which would improve diagnosis of HPV-positive head and neck (HN) tumors. Mitosoid cells were not only observed in HPV-positive benign epithelial hyperplasias but also abundantly present in subsets of pre-malignant tumors (high-grade oral epithelial dysplasia, hgOED). $P16^{INK4a}$ could be used as an HPV-surrogate marker only in Group 1 hgOED (containing diffuse mitosoid cells), but not in Group 2 (with focal mitosoid cells) and Group 3 (lacking these cells). My study revealed that E7 seropositivity complements $p16^{INK4a}$ overexpression in HNCs and a decrease in E7 serology potentially predicts patients’ response to treatment. To evaluate genetic and epigenetic changes in HPV-induced malignancy, I assessed viral DNA- integration and -methylation, and viral-induced

methylation of host tumor suppressor genes (TSGs). Since viral DNA integration predicts malignancy, my data showing integrated HPV in Group 1 and malignant tumors, suggest greater malignant transforming potential of Group 1 than the other groups. Although viral methylation is another regulatory mechanism for malignancy, the HPV epigenome was mostly unmethylated in both premalignant and malignant HN tumors. Screening 38 host TSGs identified *EREG* as a candidate gene, which may be epigenetically regulated, specifically in HPV-positive HNC.

Overall, the present study found that mitosoid cells and E7 serology in combination with $p16^{INK4a}$ overexpression are significant markers for HPV-associated head and neck malignancy. HPV DNA integration and host *EREG* gene methylation, but not viral DNA methylation, may play roles in HPV-associated head and neck carcinogenesis.

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CHAPTER I

INTRODUCTION ON HUMAN PAPILLOMAVIRUS AND HEAD AND NECK TUMORS

Human papillomavirus (HPV)

Papillomaviruses are small, non-enveloped, icosahedral DNA viruses that have a diameter of 50–60 nm. Papillomaviruses (PVs) are species-specific, and can infect different mammals, birds, and reptiles. Human papillomavirus (HPV) are PVs that infect human epithelial cells (i.e. they are epitheliotropic in nature) (1). HPV is the most common sexually-transmitted infection worldwide including the United States (2,3). HPVs are tissue-specific and normally infect either cutaneous or mucosal tissue, although some are both cutaneous and mucosotropic (1). In 1974, the German virologist Harald zur Hausen found that the skin wart virus, HPV, contributes to the development of cervical cancers (4,5). Almost 10 years later, his group isolated HPV type 16 DNA from a cervical squamous cell carcinoma (6). He was awarded the Nobel Prize in Physiology or Medicine 2008 for this discovery. Presently, HPV is known to cause virtually all cervical cancers. Recent studies have shown that HPV-associated cancers are not just limited to the anogenital region. Instead, HPV is a highly prevalent human pathogen and causes ~5% of all solid-organ cancers worldwide (7). During the past few years, there has been considerable interest in the involvement of HPV in upper aerodigestive sites and its etiologic role in causing head and neck tumors.

HPV genome

Papillomaviruses contain a circular, double-stranded DNA genome of approximately 8 kb. HPV genome contains a number of open reading frames (ORFs) classified as either early or late depending on the timing of their expression after infection. The genome also includes a long control region (LCR) or upstream regulatory region (URR), which lacks ORFs but contains *cis*-responsive elements that govern viral replication and gene expression.

Figure 1 shows a genomic map of HPV type 16. The six early ORFs [*E1*, *E2*, *E4* and *E5* (in green) and *E6* and *E7* (in red)] are expressed from promoter either p97 or p670 (in the case of HPV16) at different stages of epithelial cell differentiation. The late ORFs [*L1* and *L2* (in yellow)] are also expressed from p670. All the viral genes are encoded on plus (+) strand of the double-stranded circular DNA genome.

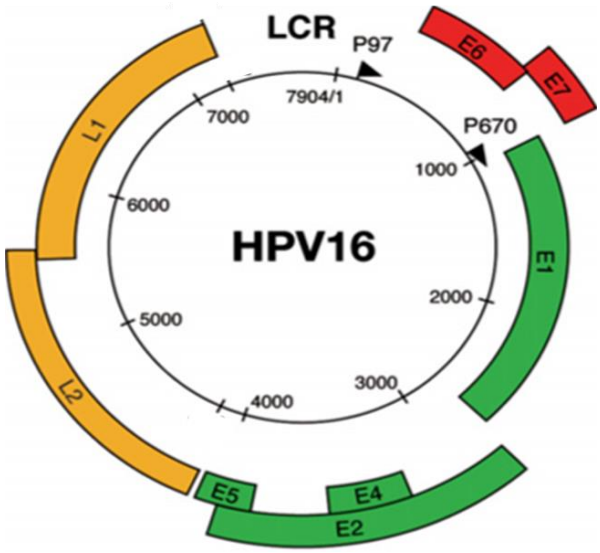

Figure 1: HPV type 16 genomic map showing early genes (*E1*, *E2*, *E4*, *E5*, *E6*, and *E7*), late genes (*L1* and *L2*) and long control region (LCR) (Copyright permission obtained from John Wiley and Sons).

The late genes encode for the structural proteins L1 (major capsid protein) and L2 (minor capsid protein), which are only expressed in productive infections. During this productive infection, HPV genomes are packaged within the capsids for viral assembly and release from keratinocytes. The early genes *E1*, *E2*, *E4-E7* are expressed prior to initiation of viral replication, but not the late genes. Among all genes, *L1* and *E1* genes are highly conserved among all papillomaviruses (8).

E1, in combination with *E2*, has a regulatory function. *E1* encodes a protein with ATPase and helicase activities and is essential for viral replication (9). *E2* gene encodes a DNA-binding protein that binds to target sequences around the viral origin and recruits the E1 helicase which in turn is involved in replicating the viral genome by recruiting host replication proteins (10). In addition to its role in viral DNA replication, the E2 protein functions as a

transcriptional repressor for the expression of E6 and E7 oncoproteins. The gene for the E4 protein overlaps the *E2* ORF (10). E4 proteins are expressed relatively late during infection before the expression of L2 and L1 and help in the maturation of viral particles (11,12). While a definite oncogenic role of E5 is not clear, it is thought to augment oncogenic activities of E6 and E7 proteins, and allow the continuous proliferation of the host cell delaying differentiation (12,13).

E6 and E7 are the main oncoproteins of high-risk HPVs. The E6 and E7 proteins bind and promote the degradation of the host p53 and retinoblastoma (pRb) tumor suppressor proteins, respectively (14) (**Figure 2**). Since most HPV-positive cancer cells harbor wild-type *TP53* and *Rb* tumor suppressor genes, these cells have intact but dormant tumor suppressor pathways due to the continuous expression of *E6* and *E7* genes (15). E6 binds the tumor suppressor protein p53 and promotes ubiquitin-mediated proteasomal degradation of p53. Hence, HPV-infected cells do not undergo apoptosis or cell cycle arrest even if DNA is damaged. E6 also activates the expression of hTERT, the catalytic subunit of telomerase and increases telomerase activity, contributing to cell immortalization (16,17). E7 is another major oncoprotein. E7 binds with pRb protein and its family proteins, p107 and p130, and disrupts their ability to form complexes with E2F transcription factors, resulting in increased expression of E2F-responsive genes, many of which are required for cell cycle progression. In addition, the E7 protein accelerates degradation of hypophosphorylated pRb family members. Inactivation of pRb by E7 can be monitored by overexpression of $p16^{INK4a}$ (**Figure 3**), which is often used as a surrogate marker of HPV infection (18).

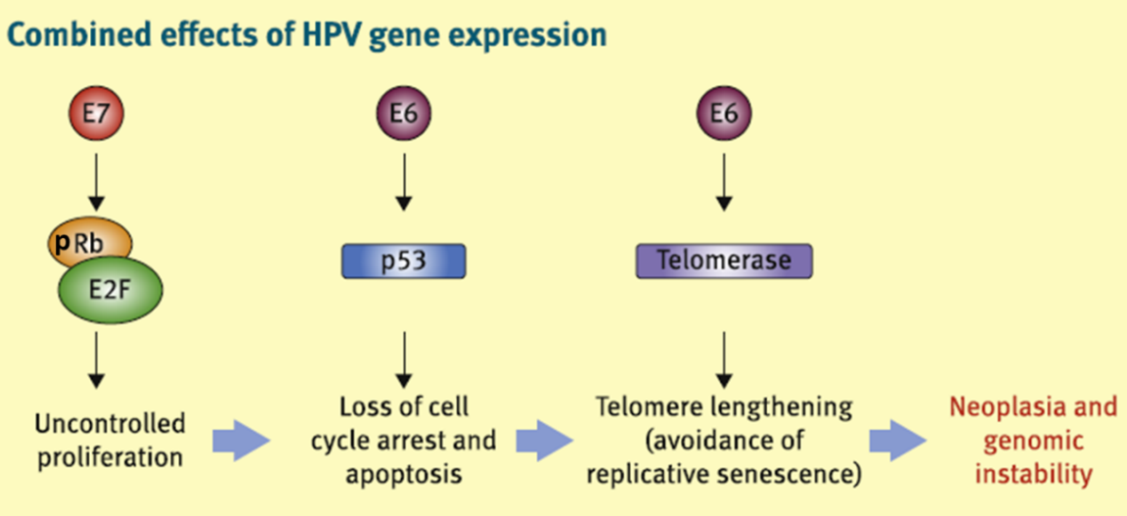

Figure 2: The combined effects of HPV E6 and E7 oncoproteins in malignant transformation. E7 and E6 inactivate tumor suppressor proteins pRb and p53, respectively. E6 also increases telomerase activity (Copyright permission obtained from Elsevier)

The long control region (LCR), also called upstream regulatory region (URR) regulates the transcription of viral genes, particularly oncogenes *E6* and *E7*. The LCR contains viral promoters, enhancers, the replication origin and binding sites for several viral and cellular transcription factors, which are important for viral replication and gene expression (10).

The expression of HPV E6 and E7 proteins is inhibited by the viral E2 protein through its interaction with the early promoter p97 located in the LCR. This promoter harbors four specific E2-binding sites (E2BSs) sharing the consensus sequence 5′-ACCG$(N)_4$CGGT-3′ (19) **(Figure 3)**. The three sites proximal to the TATA box, importantly E2BS2, E2BS3, and E2BS4, have been shown to be involved in E2-mediated repression of the promoter activity (20). Binding of E2 protein to E2BS inhibits the docking of transcription activators, such as specificity protein 1 (Sp1) and TATA-binding protein (TBP), from their binding sites, leading to a repression of *E6* and *E7* expression from the early promoter (21) (**Figure 3**). The integration of viral DNA in high-risk forms of HPV has been described as a key step in carcinogenesis since it generally results in the disruption of the *E2* ORF (22). Additionally, DNA methylation on LCR region is shown to inhibit binding of the transcriptional repressor (E2), resulting in the overexpression of viral oncoproteins, leading to malignant transformation (23,24).

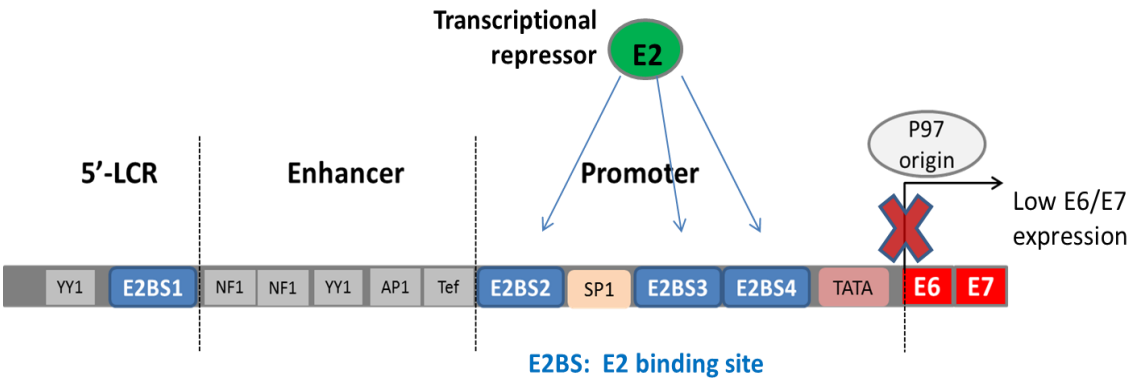

Figure 3: HPV long control region (URR) showing E2 and other transcription factor binding sites. Binding of E2 to E2BS causes repression of *E6* and *E7* expression.

Classification of HPV types

Papillomaviruses (PVs) belong to the family *Papillomaviridae.* Each PV has traditionally been referred as "type". An HPV type is defined as an HPV isolate where the nucleotide sequence of the highly conserved viral *L1* gene is at least 10% different from any known HPV type (25,26). More than 150 PV types are capable of infecting humans and thus are referred to collectively as human papillomaviruses (HPVs).

Isolates of the same HPV type are referred to as "variants" or "subtypes" when the nucleotide sequences of the *L1* gene differ by less than 10%. An HPV variant contains a unique combination of single nucleotide polymorphisms (SNPs) mainly in *E6* and/or *LCR* gene (27). Names of HPV variants are derived from the geographical origin of the human populations in which they are most prevalent. For example, HPV type 16 variants have been classified into 4 major lineages: European-Asian, African 1, African 2, and Asian American/North American (28,29).

HPV types fall into 5 genera (namely- Alpha, Beta, Gamma, Mu, and Nu) based on sequence comparisons of *L1* genes (members of the same genus share >60% nucleotide sequence identity between *L1* genes). Many types of HPV, especially those of the Beta and Gamma genera, cause asymptomatic infections without the apparent disease. The most clinically significant group is the Alpha genus which includes HPV types that infect cutaneous

or mucosal sites. Cutaneous types include HPV2 and HPV57, which cause common warts and HPV3 and 10, which cause flat warts. Mucosal HPV types are sub-divided into low-risk types and high-risk types depending on the strength of their association with carcinogenesis, **Table 1** (4,30). High-risk HPV mucosal types (such as HPV16 and 18) are highly associated with the development of precancerous and cancerous lesions while low-risk types (such as HPV6 and 11) cause benign conditions such as genital warts and respiratory papillomatosis (30). HPV is so common that nearly all sexually active people will have it at some point in their lives (2). However, only persistent infections with specific subsets of high-risk HPVs are responsible for the development of cancer (31). Among the high-risk HPV types, HPV16 and 18 are frequently found to be associated with malignancy. Between these two HPV types, HPV16 is the most prevalent carcinogenic HPV (32).

Table 1: High-risk and low-risk HPV types, based on their oncogenic potential

High-risk HPV	16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59
Putative high-risk HPV	23, 53, 66, 67, 68, 73,82
Low-risk HPV	6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, CP6108

Strategies for HPV detection, their strengths, and limitations

An ideal test for HPV would be highly sensitive, specific, cost-effective, and simple in the application so that it could be easily employed in routine diagnostic pathology practice. But none of the currently used HPV testing strategies completely satisfy all of these criteria. Depending on the technique used, the results of HPV detection can vary widely.

Polymerase chain reaction (PCR)-based method of detecting a well-conserved HPV *L1* gene is very sensitive and can detect a large number of HPV types. However, this method is relatively more expensive and is not readily available in the routine pathology laboratory, and turnaround time is also longer. Also, because of the very high sensitivity, this method requires proper handling and processing of samples to avoid a false positive result due to cross-contamination (33). Another method of detecting HPV DNA is *in situ* hybridization (ISH). Although ISH is more specific, it is less sensitive than PCR and requires technical expertise to interpret results (33,34). Also, ISH method cannot detect all HPV types using commercially

available HPV probe cocktails. Both PCR and ISH- based HPV DNA detection method are unable to detect transcriptionally active HPV (*i.e*. *E6*/*E7* expression).

As HPV-driven tumor growth is caused by E6 and E7 expression, PCR detection of E6/E7 mRNA is the best method to detect transcriptionally active HPV (34). However, this is technically challenging to be used in clinical laboratory and can't be performed effectively on formalin-fixed, paraffin-embedded (FFPE) tissues (35). A recently developed technique, RNA *in situ* hybridization is effective in FFPE tissues and is highly sensitive and specific for detecting E6/E7 mRNA (36-42). As this technique is technically cumbersome and is not yet commercially available (34), other techniques are often used in clinical practice. For example, immunohistochemical detection of $p16^{INK4a}$ expression in tumor tissue sections is commonly used in the clinic as surrogates for HPV status (43). This is based on the premise that HPV infection leads to the production of the E7 oncoprotein, which inactivates pRb tumor suppressor protein and releases $p16^{INK4a}$ gene from transcriptional inhibition thereby causing overexpression of $p16^{INK4a}$ in HPV-associated cancers, **Figure 4** (34,44,45). Immunohistochemistry (IHC) for $p16^{INK4a}$ is inexpensive, highly sensitive and can be performed in virtually any histology laboratory with very quick turnaround (34,46). Overexpression of $p16^{INK4a}$ is often used as a surrogate marker of HPV infection in oropharyngeal cancers, while its use in oral cavity cancers is not well established, even though both are subgroups of HNC (46,47). Current studies show that other non-viral mechanism can also increase $p16^{INK4a}$ expression, making it a non-specific marker for HPV infection, **Figure 5** (18,46,48). This warrants the need for reliable and appropriate biomarkers to precisely diagnose HPV status in head and neck tumors or to determine HPV-associated cancer progression.

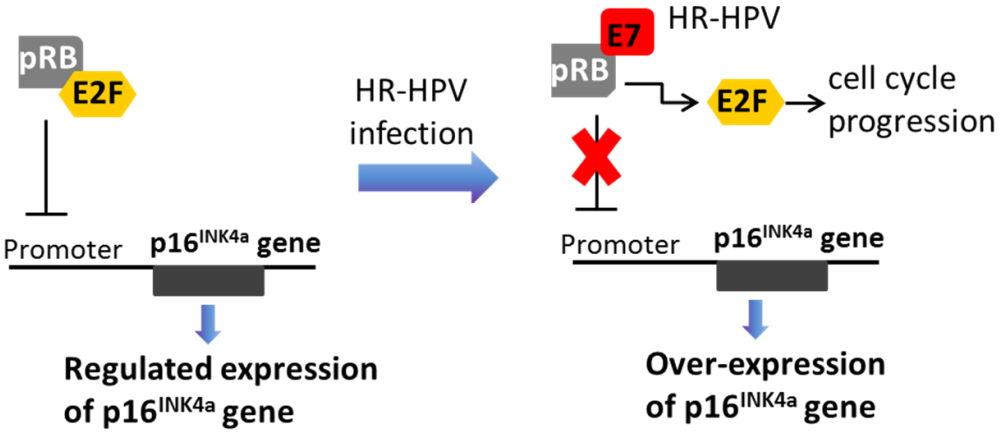

Figure 4: Presence of high-risk (HR)-HPV causes overexpression of p16^{INK4a}; as such p16^{INK4a} immunohistochemistry is used as a surrogate marker of HPV infection. The functional inactivation of pRb by HPV oncoprotein E7 results in the release of the transcriptional factor E2F from the pRb–E2F protein complex and lead to the release of the *p16^{INK4A}* gene from its transcriptional inhibition.

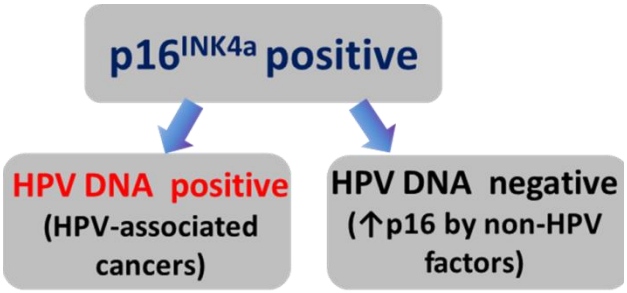

Figure 5: p16^{INK4a} is not always a surrogate marker of HPV infection. p16 ^{INK4a} is also overexpressed via non-viral mechanisms

HPV-associated clinical lesions in head and neck region

In the head and neck region, the majority of HPV-associated clinical manifestations occurs in squamous epithelium and includes benign lesions, premalignant dysplasia, and carcinomas.

1. Benign Lesions of the head and neck

There are many different types of benign oral tumors. In this study, I focused on two types of benign lesions of the oral cavity - Multiple Epithelial Hyperplasia (MEH) and squamous papilloma.

a. **Multiple epithelial hyperplasias**: MEH is an HPV-associated benign proliferation of oral squamous epithelium. It is also known as Heck's disease or focal epithelial hyperplasia (FEH) (49). It commonly occurs in Native Americans in the US but is rare among Caucasians. It usually presents in children/adolescents or HIV-infected adults and is typically associated with HPV type 13 or 32. It occurs as multiple slightly elevated and minimally keratinized papules located mainly on labial and buccal mucosa. A prominent feature of MEH is the **mitosoid cell**; an enlarged cell with a fragmented nucleus resembling atypical mitotic figure (50,51). These cells are consistently observed in MEH. Although asymptomatic and self-limited in its course, the distinction of MEH from another benign oral lesion (for example, squamous papilloma) is important since these lesions may have overlapping clinical and histologic features.
b. **Squamous papilloma:** Squamous papillomas are common benign epithelial lesions of the head and neck region. It has been suggested that many of these lesions have a viral origin but the etiology is still controversial. Several reports have demonstrated the presence of HPV in squamous papillomas (52,53). However, the presence of HPV in these types of lesions varies from one study to another (53-56). Of note, squamous papilloma may show some clinical and histologic overlap with MEH.

2. Premalignant lesions of the head and neck

Dysplasia is defined as an abnormality in maturation of epithelial cells. In other words, squamous dysplasia is the precancerous state referring to metaplastic alterations of the surface epithelium prior to the invasion of the subepithelial tissues. These changes include abnormal cellular organization, increased mitotic activity, and nuclear enlargement with pleomorphism. Although terminology varies, atypia limited to the lower one-third of the epithelium is generally referred to as mild dysplasia, atypia limited to the lower two-thirds as moderate dysplasia, and atypia involving the full thickness of the epithelium as severe dysplasia/carcinoma *in situ*. With progression, the carcinoma *in situ* breaks through the basement membrane and invades the subepithelial connective tissue (57).

In this study, I focused on high-grade oral epithelial dysplasia (i.e. severe dysplasia/carcinoma *in situ*). These are pre-cancerous lesions which often develop into oral

squamous cell carcinoma (SCC), a subset of head and neck SCC (58). Studies of HPV-associated cervical carcinogenesis have looked at clearly defined stages, including asymptomatic infection, cervical intraepithelial neoplasia (CIN) stages I to III, and cancer (59), whereas HNC studies conducted to date have mainly focused on primary cancers. As such, there are few studies evaluating the presence of HPV in the pre-cancerous lesions.

3. Head and neck squamous cell carcinoma

The majority of the cancers in the head and neck region develop from thin, flat cells called squamous cells. Cancer that starts in the layer of squamous cells is called squamous cell carcinoma. Head and neck squamous cell carcinoma (HNSCC) or head and neck cancer (HNC) is the sixth most common cancer and eighth leading cause of cancer-related deaths worldwide and it represents about 3% of all cancers in the United States (60,61). It develops in the mucosa of the upper aerodigestive sites including oral cavity, oropharynx, hypopharynx or larynx (62). Because of anatomic locations associated with the ability to look, talk, eat and breathe, these cancers can highly affect the quality of life. There are two major risk factors for the development of HNC: exogenous carcinogens (namely tobacco and alcohol exposure) and infection with HPV. Cancer-related death ranges widely from 20-60% in patients presenting with locally advanced forms of HNC. While many tobacco-related cancers are on the decline owing to decreases in smoking among Americans, the number of HPV-related HNC cases appears to be increasing (62).

HPV-associated and unassociated HNCs display differences in clinical outcome: patients with HPV-positive HNC respond better to chemo/radiotherapy and have higher overall survival rates (63,64). Because of this, many clinicians advocate for a reduction in treatment intensity for patients with HPV-induced cancers to minimize post-treatment morbidity (65). However, a reduction in treatment intensity risks the possibility of under-treatment, tumor progression, and metastasis in patients who are HPV negative and may have responded to current high-intensity treatments (66). So, a detailed understanding of HPV status of HNC patients is warranted to determine appropriate therapies.

The molecular profiles of HPV-positive HNC are distinct from those of HPV-negative cancers. In HPV-positive cancers, HPV oncoproteins E6 and E7 promote the degradation of

p53 and pRb tumor suppressors, respectively, and $p16^{INK4a}$ protein is upregulated. Overexpression of $p16^{INK4a}$ protein is the result of the effect of the viral E7 protein in suppressing the function of pRb. In contrast, in HPV-negative HNC, *TP53* is frequently mutated and $p16^{INK4a}$ is under-expressed. HPV-negative HNCs are reported to accumulate a large number of cellular mutations compared to HPV-positive HNCs (67,68). These molecular differences are suggested to be the reasons behind better prognosis of HPV-positive HNC. However, the specific mechanisms responsible for improved prognosis/survival in HPV-positive patients have not been fully elucidated. For these reasons, the knowledge of differential mechanisms distinguishing HPV-positive and -negative HNC is very important; which may underlie the molecular basis of improved prognosis for HPV-induced cancer as well as provide opportunities for new therapeutic development.

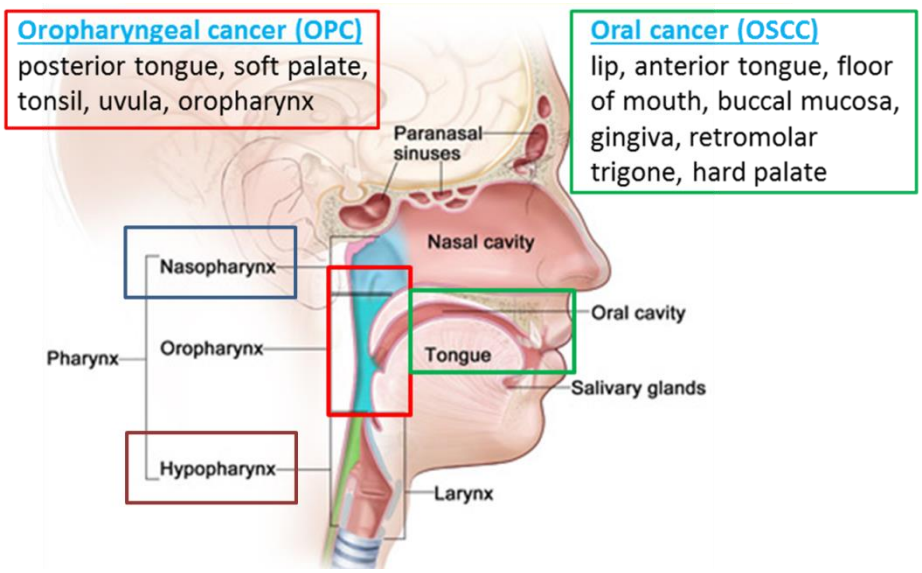

Figure 6: Pictorial representation of the anatomy of the head and neck cancer showing different subgroups (Copyright permission obtained from Terese Winslow LLC, U.S. Govt).

There are different subgroups of HNCs based on anatomic locations (69) (**Figure 6**). HNCs driven by tobacco and alcohol use can occur anywhere throughout the upper aerodigestive tract while cancers driven by HPV appear to be mostly localized to the oropharynx (34).

Oropharyngeal squamous cell carcinoma (OPSCC or OPC): The incidence of OPC is increasing as smoking-related cancers decrease (43). This increase is due to HPV, which has emerged as the primary etiologic factor in OPC, surpassing tobacco and alcohol as risk factors in this type of HNC (70). Cohort studies from the 1990s suggested that approximately 50% of oropharyngeal cancers were attributable to HPV, while more recent studies suggest that HPV accounts for 70 to 80 % of cases in North America and Europe (71).

An oropharyngeal subsite of the head and neck region most affected by HPV includes the soft palate, base of tongue, tonsils, throat walls and oropharynx (72). More specifically, HPV infection is most commonly associated with the palatine and lingual tonsils (62).

Oral cavity squamous cell carcinoma (OCSCC): The possible role of HPV in the etiology of OCSCC is currently not well-defined. The role of HPV in carcinogenesis of the oral cavity is further obscured by discordant findings between $p16^{INK4a}$ IHC and HPV testing in these tumors. While HPV is detected in relatively few OCSCC tumors, the majority of these tumors exhibit overexpression of $p16^{INK4a}$ (58,73). Reports show that the majority of $p16^{INK4a}$ - positive OCSCC were negative for HPV (74-76), suggesting that $p16^{INK4a}$ is not always an absolute marker to detect HPV. In OCSCC, non-viral mechanisms appear to stimulate $p16^{INK4a}$ overexpression, making it relatively non-specific and therefore an increasingly unreliable surrogate marker for HPV detection in OCSCC (74-76).

Other HNC types are ***nasopharyngeal, laryngeal*** and ***hypopharyngeal*** carcinoma where tobacco and alcohol are the main risk factors/ primary etiologic agents. These types of cancers in head and neck region are found to be less likely to be associated with HPV (77).

Biology of HPV infection

HPV infections are most frequently seen in the epithelia of the uterine cervix and head and neck region. The biology of HPV infection in cervical cancers has been well-documented but there is less information available for HNCs.

In cervical cancer, HPV virions attach to the basement membrane and preferentially infect basal cells in cervical transformation zone, located in the boundary between the

squamous epithelium of ectocervix and the columnar epithelium of endocervix (called squamocolumnar junction) (4). Viruses are known to use the host cell machinery for replication. As the HPV-infected keratinocytes differentiate and move toward the epithelial surface, different viral genes are expressed, allowing high viral genome amplification and the expression of the late region genes that encode the viral capsid proteins necessary to complete viral life cycle (78). As the cells reach the surface, the HPV episomes are packaged within the capsids for final viral assembly and release (called lytic infection) (79). Abnormal cell growth in the lower 1/3 of the cervical epithelium is classified as HPV infection or cervical intraepithelial neoplasia 1 (CIN1) and 2/3 of the way from the basal layer as CIN2. These CIN1 and CIN2 lesions typically disappear within a few months by the immune system (80). However, the persistent infection causes abnormal cell growth to penetrate the entire thickness of cervical epithelium causing CIN3 or carcinoma *in situ*. When abnormal cells invade the basal membrane and spread into the tissues beneath, the condition becomes cervical cancer (81).

The biology underlying HPV infections in the head and neck region is largely unknown, particularly in areas other than the oropharynx. Oropharyngeal tissues do not contain squamocolumnar junctions. However, the palatine and lingual tonsils are lined with stratified squamous epithelium and contain numerous tonsillar crypts that extend through the full thickness of the tonsil (82,83). These crypts are lined with specialized reticular epithelium that has a porous basal cell layer and disrupted basement membranes. It has been proposed that the structural porosity of the tonsillar reticulated crypt epithelium allows HPV access to the basement membrane and infection of basal cells without the associated trauma observed in cervical cancer (57).

Since tumorigenesis in oropharynx often occurs deep within the crypts, it is difficult to identify premalignant precursors on the tissue surface (47). Unlike oropharyngeal cancers (OPC), oral squamous cell carcinoma (OSCC) often develops from a clinically apparent leukoplakia (84) that shows microscopic evidence of oral epithelial dysplasia. Therefore, oral cavity pre-malignant lesions may provide an *in vivo* model to study the development of head and necks involving an HPV etiology.

Molecular events associated with HPV malignancy

Studies in cervical cancer, the most widely characterized HPV-associated malignancy, indicate that HPV-driven malignant conversion is associated with specific molecular events. HPV-associated carcinogenesis requires continuous overexpression of the two main viral oncoproteins E6 and E7, which interact with many cellular proteins leading to the induction and maintenance of a transformed phenotype in infected cells (15). *E6* and *E7* expression are regulated by the viral E2 protein through the early promoter (termed p97 for HPV16) located in the LCR of the viral genome. Overexpression of *E6* and *E7* is caused either by disruption of *E2* gene via HPV genome integration or by inhibition of E2 protein binding to the LCR via methylation of viral DNA. Therefore, the integration and methylation of viral DNA are considered as two main regulatory mechanisms for malignant transformation, mainly in the cervical region (85). Additionally, DNA hypermethylation of host genes was frequently detected in advanced forms of cervical cancer (86-90). In contrast, molecular events culminating in malignant transformation of the HPV-infected head and neck cells are largely unknown.

HPV DNA integration

HPV DNA may exist in the episomal (extra-chromosomal) form and/or become integrated into the host genome (**Figure 7A**). HPV DNA integration into the host genome has been considered to be an important molecular event during HPV-induced cervical carcinogenesis (91,92), (**Figure 7B**). In most invasive cervical cancers, high-risk HPV genomes are integrated into the host genome mainly via mechanisms that disrupt the *E2* reading frame (93-95). Lack of E2 results in up-regulation of the viral oncogenes *E6* and *E7*, thereby promoting tumorigenesis (**Figure 7**). In addition to invasive cervical cancers, integration of HPV DNA was also found in premalignant cervical intraepithelial neoplasia (CIN2/3). In contrast, in benign and low-grade cervical lesions the predominant form of HPV DNA is episomal (96,97). Accordingly, different patterns of HPV DNA integration are currently used to monitor cancer progression in cervical cancer patients (98,99).

While viral integration is thought to play an important role in cervical carcinogenesis (92), the relevance of viral DNA integration is controversial in head and neck tumors (100). Some studies suggest that viral DNA integration in the head and neck sites plays an important role in carcinogenesis (83,101-105), while other studies suggest that episomal HPV alone contributes to the development of most HNCs, in contrast to what is observed in cervical cancers (100,106-108). Therefore, more research needs to be done to better understand the role of HPV DNA integration in head and neck malignancy.

HPV DNA methylation

Methylation of DNA is facilitated by a family of DNA methyltransferases (DNMTs) that catalyze the addition of methyl group to cytosine located in CpG dinucleotides. Like the human genome, the HPV genome is subject to epigenetic regulation through alterations in DNA methylation. There are 15 CpG sites in the LCR of HPV16 genome (**Figure 8**). Methylation of these 15 CpG sites (mainly 5 sites) inhibits the binding of E2 protein to E2BS, in turn, leads to an overexpression of *E6* and *E7* from the early promoter, **Figure 8**. Methylation of the five CpGs located in the E2BS3 and E2BS4 and Sp1-binding site (Sp1BS) has been suggested as biomarkers for cervical cancer progression (23,24). Such hypermethylation has been reported in cervical cancers or high-grade cervical dysplasia compared with low-grade CIN (23,109,110). But it is unknown whether or not viral methylation in HNC has a similar association with malignancy, although recent studies have suggested that there may be some similarities between cervical cancers and HNCs with respect to viral methylation (111). In contrast, a large study reported hypomethylation of the LCR of the viral genome in oropharyngeal cancers (112). Therefore, more research remains to be done to clarify a role of viral methylation in HPV-associated HNCs.

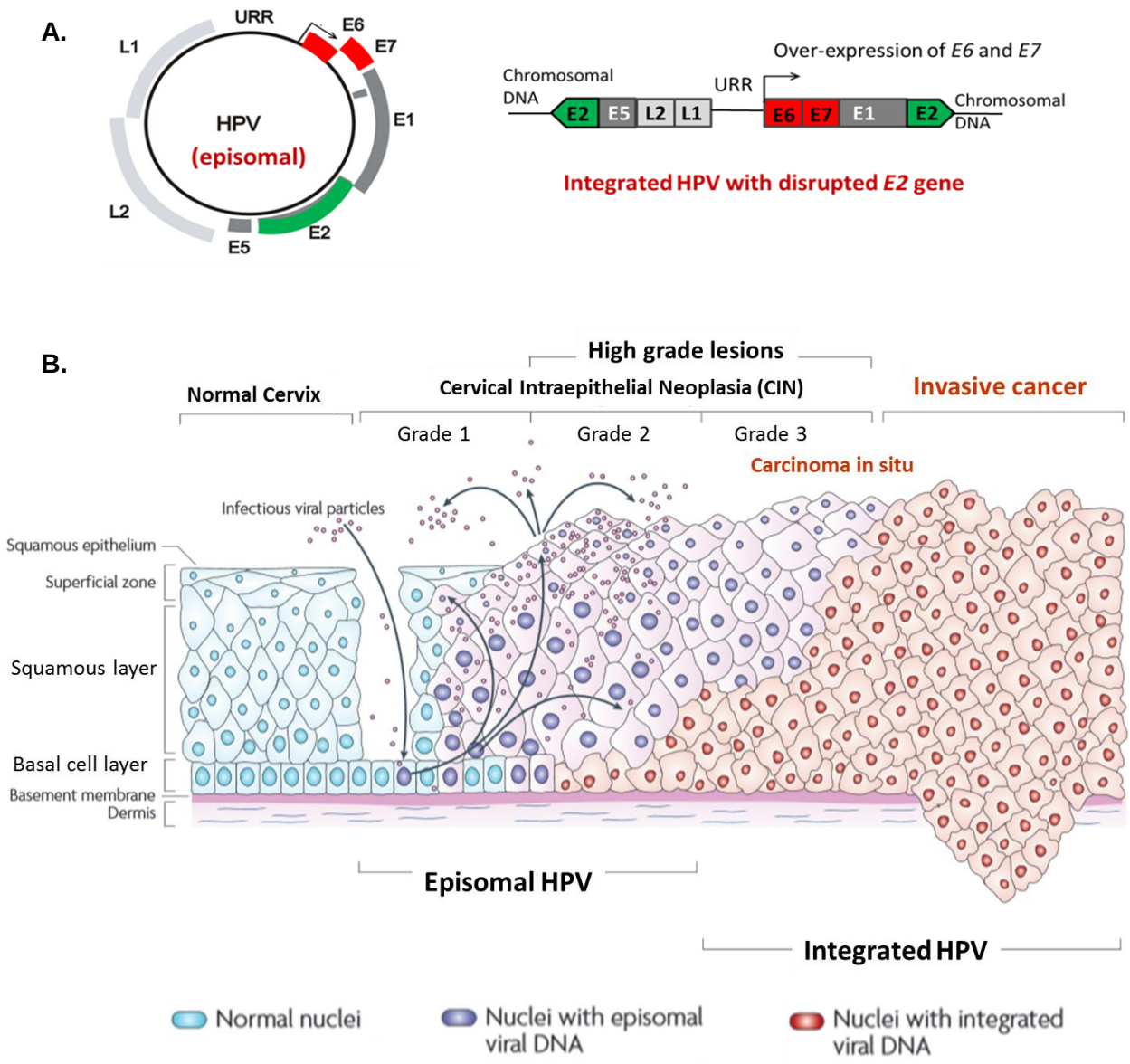

Figure 7: (A) Episomal and integrated forms of HPV DNA. Integration of HPV DNA is associated with loss or disruption of E2, and subsequent upregulation of E6 and E7 oncogene expression. **(B)** HPV-DNA integration- mediated progression to cervical cancer. HPV infects basal cells in the cervix. Low-grade cervical intraepithelial lesions (CIN) support productive viral replication. The progression to high-grade CIN and invasive cancer is associated with the integration of the HPV genome into the host chromosomes (red nuclei) from episomal HPV (dark blue nuclei). (Copyright permission obtained from Nature Publishing Group)

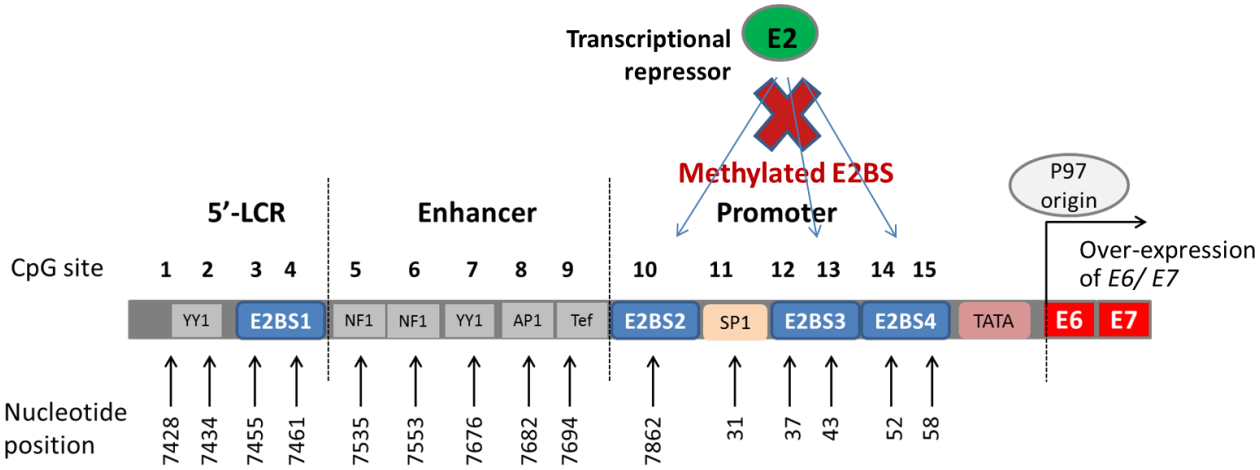

Figure 8: Upstream regulatory region of HPV16. Methylated E2BS inhibits the binding of E2 transcriptional repressor and in turn, increases *E6* and *E7* transcription. Fifteen CpG sites are shown along with their respective nucleotide positions using an Asian-American variant of HPV16 (Genbank accession number: AF402678.1) as a reference sequence.

Host genomic epigenetic changes

DNA methylation is one of the most intensely studied epigenetic modifications in humans. In normal cells, DNA methylation plays a role in maintaining genome stability and in regulating the expression of genes involved in signal transduction cascade pathways, cell cycle regulation, angiogenesis, apoptosis, and DNA repair (113). Methylation of DNA typically occurs on cytosines located in CpG dinucleotides, which are usually concentrated in large clusters called CpG islands. The methylation of cytosine residues is mediated by a class of enzymes called DNA methyltransferases (DNMTs) that catalyze the transfer of the methyl group from S-adenosyl-methionine to the carbon 5 position of cytosine (**Figure 9A**). DNA methylation often leads to gene silencing after chromatin structure remodeling (114). Methyl-CpG-binding proteins (MBD) associates with methylated CpG that, in turn, recruits histone deacetylases (HDAC). As a consequence, chromatin becomes compacted and gene expression is inhibited (113), (**Figure 9C**).

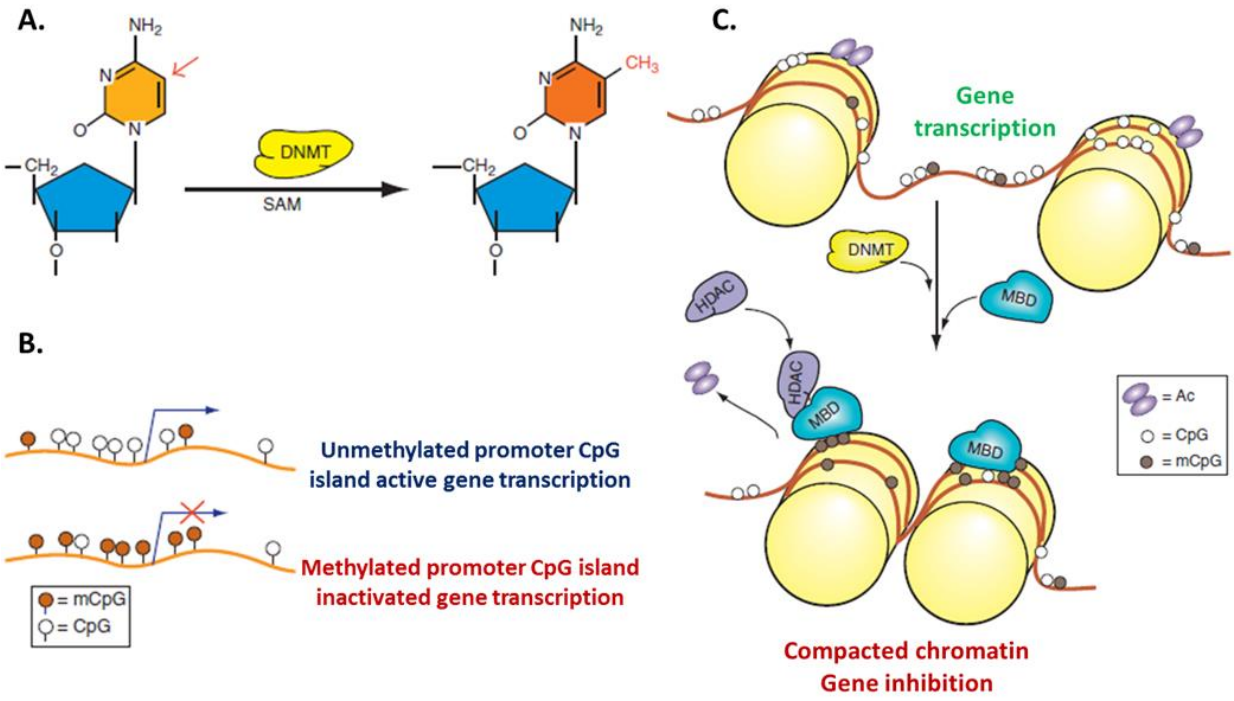

Figure 9: Mechanism of gene silencing mediated by DNA methylation. **A)** Cytosine methylation catalyzed by DNMT in the presence of S-adenosyl-methionine (SAM). **B)** Unmethylated CpGs within the promoter region allows gene expression while methylated CpGs causes silencing of genes. **C)** DNA methylation occurs through the recruitment of methyl-CpG-binding proteins (MBD) and histone deacetylases (HDAC) resulting in compacted chromatin and gene inhibition (Copyright permission obtained from Elsevier)

Aberrant methylation of gene promoters, particularly of tumor suppressors can lead to their transcriptional repression, thereby resulting in the pro-carcinogenic environment (115,116). Recent studies have identified epigenetic alteration, most importantly aberrant DNA methylation, as a possible cause of progression of various cancers (116-118). In addition, recent evidence revealed that HPV16 induces DNA methylation in infected cells by up-regulating DNMTs (DNMT1 and DNMT3a) (119,120). Different studies related to epigenetic alterations have been largely investigated in cervical cancers, where hypermethylation of viral and host genes was frequently detected in advanced cancers (86-90). In addition to cervical cancers, aberrant promoter hypermethylation also has been identified in the progression of HNCs (121,122). However, the differences in epigenetic anomalies between HPV-positive and HPV-negative HNCs are not well-defined (123-125). A

better understanding of these abnormalities will help to know the epigenetic mechanism regulated by the presence of HPV in HNCs.

Dissertation Overview

Overall Goal

The information summarized above suggests that understanding molecular events involved in HPV-associated head and neck cancer progression will have diagnostic and prognostic significance. Therefore, it is crucial to study HPV-associated potential biomarkers and molecular events that occur early in head and neck cancer progression in order to enhance detection. The ability to document an HPV etiology for HNCs can affect therapeutic decision-making, factor into a patient's long-term prognosis, and identify new therapeutic targets.

Hypothesis and Research Aims

The overall aim of my dissertation is to identify potential biomarkers and ascertain underlying molecular mechanisms associated with HPV-positive head and neck malignancy. I hypothesized that head and neck tumors display distinct HPV-associated biomarkers and oncogenic mechanisms for malignancy, which differ from that of HPV-negative head and neck tumors.

My dissertation includes three major areas of investigation outlined in chapters 2, 3, and 4. In chapter 2**,** I analyzed tumors of the head and neck for the presence of HPV and found that HPV-positive tumors were molecularly and histologically distinct. In chapter 2 and 3, I identified potential clinical biomarkers for the detection of HPV-associated head and neck malignancies. Finally, in chapter 4, I focused on the role of HPV DNA integration, HPV methylation and host gene methylation in tumors of the head and neck to identify genetic and epigenetic mechanisms involved in HPV-mediated malignancy.

CHAPTER II

HISTOLOGIC VARIATION IN HEAD AND NECK TUMORS WHEN ASSOCIATED WITH HUMAN PAPILLOMAVIRUS

Chapter Overview

Recently, HPV has been found to be increasingly implicated as a causative agent in different types of tumors in the head and neck region. In this chapter, I determined the involvement of HPV in pathologically distinct tumor specimens of the head and neck and identified potential histological biomarkers which could be used to detect HPV infection.

This chapter is divided into **three sub-headings**, based on the study conducted on different head and neck tumor specimens:

1. Benign oral lesions (126)
2. Premalignant oral lesions (127)
3. Carcinoma of head and neck

1. Benign oral lesions

Introduction

Multifocal epithelial hyperplasia (MEH) or Heck's disease is a benign oral condition that occurs in Native American children/adolescents or HIV-infected adults in the United States but is rare among Caucasians and HPV-negative adults. It is typically associated with low-risk HPV infections typically involving HPV type 13 or 32 (49). Histologically, MEH lesions

[A study of benign oral lesions was published in 2015 (126) and copyright permission was obtained from Elsevier to reuse in this dissertation. A manuscript regarding premalignant lesions is currently under revision for publication in *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology*; an abstract published in 2016 (127)]

are characterized by mild hyperkeratosis and thickening of the epithelium with elongated rete ridges. A prominent feature is the presence of a mitosoid cell, an enlarged cell with atypical/fragmented nucleus resembling a mitotic figure (50,51). Mitosoid cells are atypical mitotic figures, which are usually seen in well-differentiated upper epithelial layers of hyperplastic oral mucosa (128).

Recently, we have identified oral lesions that mimic MEH microscopically but occur in adult Caucasian patients who are not known to be HIV positive. Oral squamous papillomas are another HPV-associated benign lesion which shows clinical & histological overlap with MEH but do not occur in the clinical setting expected for Heck's disease (*i.e.* children/ adolescents) (52). It is important to distinguish different forms of MEH from one another and from squamous papilloma. HPV genotyping could potentially contribute to differentiating between these different lesions. Herein, I performed HPV genotyping of oral lesions microscopically consistent with MEH and compared these results with those derived for squamous papilloma.

Experimental Procedures

Patient's biopsy sample collection: This study was approved by the Institutional Review Board (IRB) at the University of Louisville. MEH (N=22, 17 patients) and squamous papilloma (N=9, 9 patients) samples were selected based on well-defined histologic criteria. Histologically, MEH should have thickening of the epithelium (acanthosis) with elongated rete ridges and mitosoid cell(s) within the well-differentiated upper epithelial layer called spinous cell layer (**Figure 10**). The histologic inclusion criteria for squamous papilloma were: keratinized stratified epithelium with finger-like surface projection and without the presence of any mitosoid cell(s).

Genomic DNA extraction and HPV detection: Formalin-fixed, paraffin-embedded (FFPE) specimens were deparaffinized using xylene, followed by an ethanol wash. DNA was extracted using DNAeasy Blood & Tissue kit (Qiagen, USA) as per manufacturer's instructions. HPV DNA detection was performed by PCR amplification using conventional degenerate MY09/11 and GP5+/GP6+ consensus primers to a well-conserved HPV L1 gene. Both primers can detect a wide range of HPV types. The reaction conditions were as follows:

50-100ng of template DNA, HiFi PCR buffer, 50mM MgSO4, 10mM dNTPs, 20µM of each primer and 1U of HiFi Taq polymerase (Invitrogen) in a total volume of 20µl reaction mixture. PCR reaction consisted of an initial 94°C incubation for 2 min, followed by 44 cycles of denaturation at 94°C (45 sec), annealing step at 56°C (for MY product) or 48°C (for GP product) for 45 sec, and 68°C (45 sec) and a final elongation step of 1 min at 68°C in a MJ Research Peltier Thermal Cycler PTC-200. The PCR amplified products were analyzed by electrophoresis on a 3% agarose gel stained with ethidium bromide and observed under ultraviolet light. PCR products on gels were purified using QIAquick Gel Extraction Kit (Qiagen, USA) and sent for DNA sequencing to the DNA Core Facility at the University of Louisville. Sequences obtained were analyzed using NCBI BLASTn database and MegAlign®. DNASTAR software (Version 12.0, Madison, WI), and type of HPV was identified. The quality of the extracted DNA was checked by amplification of internal control (β-globin). For a patient who showed two types of HPV in his/her lesions, HPV-type specific PCR was employed (which detects the presence of the E7 region of specific HPV type) to test the co-infection of HPV6 and 32 in lesions.

Statistical Analysis: Distributions of the discrete lesion and subject characteristics were summarized by frequency and percentage. Fisher's exact tests were applied to examine differences between the MEH cases and squamous papilloma. These studies also assessed the types of HPV associated with the MEH lesions in this patient cohort. Means, standard deviation, and p-values from Student's t-tests were reported. All data analyses were conducted by SAS /STAT® Software (Version 9.4.,SAS Institute Inc., Cary, NC, USA).

Results

Clinical and histologic features of MEH and squamous papilloma lesions

MEH lesions presented as a well-circumscribed, white plaque or nodule with a finely stippled/papillary surface. There was significant differences between MEH and squamous papilloma cases in distributions of the number ($p=0.006$) and site ($p<0.0001$) of lesions (**Table 2**). According to my data, MEH lesions occurred more frequently at multiple anatomic sites than squamous papillomas (66.7% vs. 0.0%). The MEH cases were less likely to appear in

the soft palate/oropharynx than squamous papillomas (0.0% vs. 55.6%) and were more likely to appear in both keratinized oral sites and the labial/buccal mucosa than squamous papillomas. Of the MEH lesions that were HPV positive 13/19 (68.4%) occurred on the labial/buccal mucosa.

MEH lesions presented with characteristic histologic features: acanthosis with elongation of the rete ridges (**Figure 10a**) and occasionally altered nuclei typical of mitosoid cells in the spinous cell layer (**Figure 10b**). Squamous papilloma cases, on the other hand, showed multiple fingerlike surface projections covered by hyperkeratotic stratified squamous epithelium supported by fibrovascular connective tissues. While typical mitotic cells are often seen in these lesions they are confined to the basal and parabasal cell layers (**Figure 11b**).

HPV status of MEH lesions and squamous papillomas

Figure 12 shows images of agarose gels of 9 representative samples. When the amplicons of MY and GP primers were sequenced, 8 cases were HPV6, and 11 cases were HPV32, while one sample was HPV40. Other samples, which did not produce any bands and of an inappropriate size for both MY and GP amplicons, were classified as negative for HPV DNA. Altogether, 19/22 MEH samples and 1/9 squamous papilloma cases were HPV positive. The presence of HPV differed significantly between MEH and squamous papilloma specimens (p=0.0002, **Table 2**). The MEH lesions were more likely to have HPV than the squamous papillomas (86.4% vs. 11.1%), especially those involving HPV type 6 (36.4% vs. 0%) and type 32 (45.4% vs. 11.1%).

HPV status in MEH lesions

Analysis of MEH lesions revealed that the number of oral lesions was significantly different depending on the HPV type detected (p=0.002). HPV32 lesions occurred significantly more often as multiple lesions than HPV6, HPV40, and HPV negative lesions (**Table 3**). Marginal differences in race, HIV status, and duration of the lesion were observed across HPV types in MEH cases. A higher proportion of African Americans were observed with HPV32 lesions than HPV6, HPV40, and HPV-negative lesions. Subjects who were reported as HIV positive had HPV32 but none of the other HPV types. In addition, HPV32 was most frequently associated with lesions occurring for a longer duration.

I examined more than one lesion, biopsied from different sites, per patient from four MEH subjects. All lesions were biopsied from different sites on the same day except in the case of patient #15. Three MEH patients were positive only for HPV32 in all of their lesions. In contrast, one patient (ID #15) with 3 lesions had HPV6 in two lesions and HPV32 in the other, as detected by MY/GP primer-based PCR. In one instance (case #35) sequencing based on HPV-type specific primers (HPV6 E7 and HPV32 E7) revealed co-infection with HPV6 and 32.

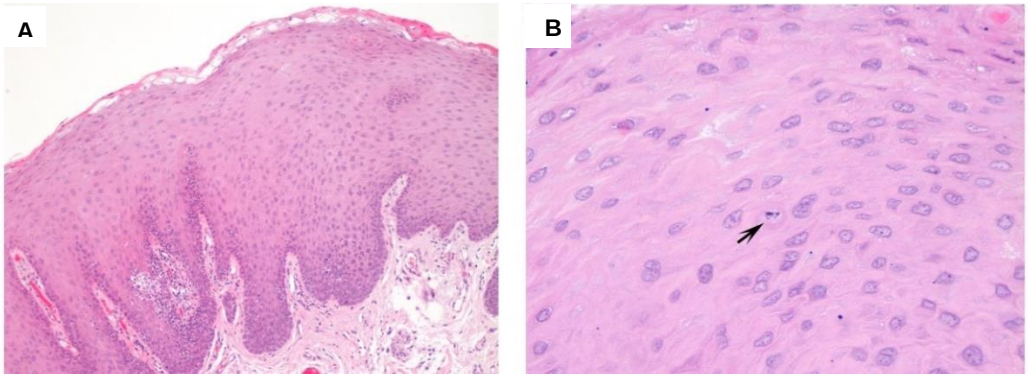

Figure 10: Photomicrographs of MEH lesion showing (**A)** acanthosis and elongation of anastomosis of the rete ridges, H&E 100X; and **(B)** a mitosoid cell (arrow) in the spinous cell layer, H&E 200X magnification

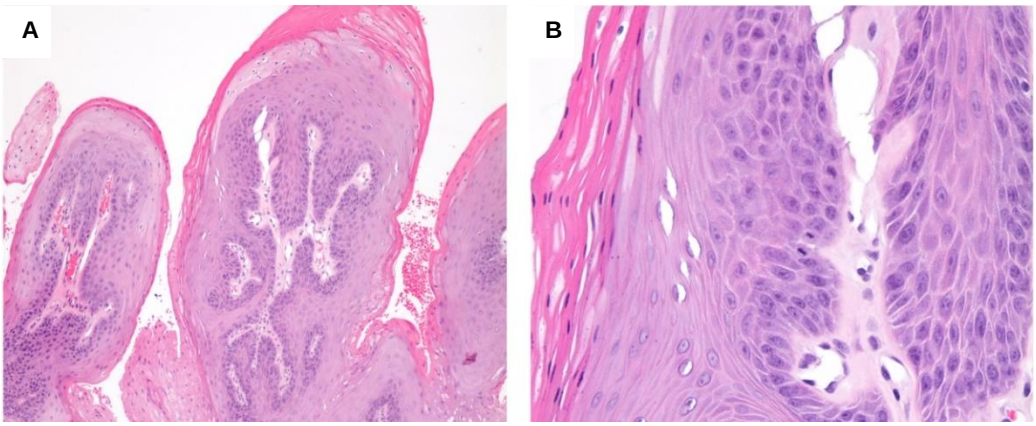

Figure 11: Photomicrographs of squamous papilloma showing **(A)** the characteristic hyperkeratosis and finger-like projections, H&E 100X, and **(B)** normal mitoses in the basal and parabasal cell layers, H&E 400X magnification

Table 2: Clinical variables of MEH cases and squamous papillomas

Variable*	Squamous papillomas (9 Subjects, 9 Lesions)		MEH Lesions (17 Subjects, 22 Lesions)		p-value
	n	%	n	%	
Gender					
Female	3	33.3	5	29.4	1.00
Male	6	66.7	12	70.6	
Race					
African American	1	11.1	3	17.6	1.00
Caucasian	8	88.9	12	70.6	
Unknown*	0	0.0	2	11.8	
Age (Mean ± SD)	46.9 ± 23.9		52.4 ± 16.9		0.50
<40 years	3	33.3	5	29.4	1.00
40-59 years	4	44.4	7	41.2	
≥60 years	2	22.2	5	29.4	
HIV					
Negative	9	100.0	13	76.5	0.28‡
Positive	0	0.0	3	17.6	
Unknown*	0	0.0	1	5.9	
HPV					
Absent	8	88.9	3	13.6	0.0002†
6	0	0.0	8	36.4	
32	1	11.1	10	45.5	
40	0	0.0	1	4.5	
Number of Lesions					
Single	7	100.0	7	33.3	0.006‡
Multiple	0	0.0	14	66.7	
Unknown*	2	22.2	1	4.5	
Site of Lesion					
Soft palate	5	55.6	0	0.0	<0.0001
Keratinized oral sites	0	0.0	4	18.2	
Labial/buccal mucosa	0	0.0	14	63.3	
Tongue/floor of mouth	4	44.4	4	18.2	
Duration of Lesion					
≤6 months	2	22.2	6	27.3	0.47‡
>6 months	0	0.0	6	27.3	
Unknown*	7	77.8	10	45.5	
Size of Lesion					
≤3 mm	6	66.7	10	45.5	0.76‡
4-6 mm	2	22.2	6	27.3	
>6 mm	1	11.1	5	22.7	
Unknown*	0	0.0	1	4.5	

*unknown: clinical information that could not be retrieved from biopsy requisition sheets

†Negative (absent) vs. positive (HPV6, 32, and 40)

‡ The unknown value was omitted for analysis.

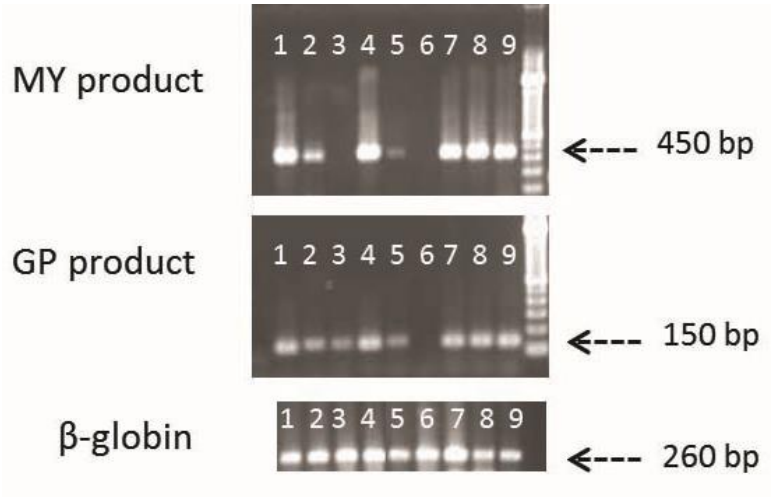

Figure 12: HPV DNA detection in benign oral samples by PCR using MY and GP primers showing amplified products of 450bp and 150bp, respectively. Amplified β-globin represents the loading control to check the quality of extracted DNA.

MEH lesions in HIV-positive subjects

In the MEH group, 3 subjects (17.6%) had HIV compared to 0 subjects in the squamous papillomas group, although no significant difference was observed between HIV-status and the groups, **Table 2**. HIV status was marginally associated with HPV genotyping (p=0.07, (**Table 3**). All 3 HIV-positive MEH patients (total of 5 lesions) were HPV32 positive (**Table 3**). Therefore, HPV6 was not detected in known HIV positive patients. Interestingly, I found that one MEH case in an HIV positive patient was on the skin side of the commissure. To our knowledge, HPV32 has never been reported in the skin. Since clinical pictures of specimens were not available to rule out the possibility that skin infection might be an extension from the oral mucosal side, I cannot substantiate my result as the first report of HPV32 on the skin surface.

Table 3: Clinical variables of MEH lesions based on HPV status

Variable	HPV-negative MEH		HPV-positive MEH		HPV-positive MEH cases						p-value*
					HPV6		HPV32		HPV40		
	n	%	n	%	n	%	n	%	n	%	
Gender											
Female	2	66.7	6	31.6	2	25.0	4	40.0	0	0.0	0.62
Male	1	33.3	13	68.4	6	75.0	6	60.0	1	100.0	
Race											
African American	0	0.0	5	26.3	0	0.0	4	40.0	1	100.0	0.08
Caucasian	3	100.0	12	63.2	6	75.0	6	60.0	0	0.0	
Unknown	0	0.0	2	10.5	2	25.0	0	0.0	0	0.0	
Age											
<40 years	0	0.0	8	42.1	5	62.5	3	30.0	0	0.0	0.13
40-59 years	1	33.3	7	36.8	3	37.5	4	40.0	0	0.0	
≥60 years	2	66.7	4	66.7	0	0.0	3	30.0	1	100.0	
HIV											
Negative	3	100.0	13	68.4	7	87.5	5	50.0	1	100.0	0.07
Positive	0	0.0	5	26.3	0	0.0	5	50.0	0	0.0	
Unknown	0	0.0	1	5.3	1	12.5	0	0.0	0	0.0	
Number of Lesions											
Single	2	66.7	5	26.3	4	50.0	0	0.0	1	100.0	0.002
Multiple	0	0.0	14	73.7	4	50.0	10	100.0	0	0.0	
Unknown	1	33.3	0	0.0	0	0.0	0	0.0	0	0.0	
Site of Lesion											
Skin	0	0.0	1	5.3	0	0.0	1	10.0	0	0.0	0.36
Keratinized oral sites	2	66.7	2	10.5	1	12.5	1	10.0	0	0.0	
Labial/buccal mucosa	0	0.0	13	68.4	6	75.0	6	60.0	1	100.0	
Tongue/floor of mouth	1	33.3	3	15.8	1	12.5	2	20.0	0	0.0	
Duration of Lesion											
≤6 months	1	33.3	5	26.3	4	50.0	0	0.0	1	100.0	0.07
>6 months	1	33.3	5	26.3	1	12.5	4	40.0	0	0.0	
Unknown	1	33.3	9	90.0	3	37.5	6	60.0	0	0.0	
Size of Lesion											
≤3 mm	1	33.3	9	47.4	3	37.5	6	60.0	0	0.0	0.20
4-6 mm	0	0.0	6	31.6	4	50.0	2	20.0	0	0.0	
>6 mm	2	66.7	3	15.8	1	12.5	1	10.0	1	100.0	
Unknown	0	0.0	1	5.3	0	0.0	1	10.0	0	0.0	

*****analyses between HPV types and variables

Discussion

I studied two types of benign oral lesions (MEH, multifocal epithelial hyperplasia, and squamous papilloma) and found a significant difference in HPV infection in these lesions. The most important finding from my study is the demographic presentation of MEH lesions. Although seen throughout the world, MEH is predominantly seen in Native Americans children/adolescents or HIV-infected adults and is typically associated with HPV types 13 and 32. However, my study demonstrated a novel presentation of MEH with HPV6, 32 and a unique type HPV40 in middle-aged to older (31-82 years of age), HIV-negative Caucasian adults. Of note, the most widely and frequently published HPV in MEH, i.e. type 13, (49) was not detected in any samples in this study. Future HPV detection protocols should include HPV32 as it may be currently overlooked. Another benign lesion *i.e.* squamous oral papilloma, which showed histologic overlap with MEH, was found to be mostly HPV negative.

Interestingly, different HPV types were noted in a patient with recurrent lesions. This patient had an initial HPV6-positive lesion, followed 15 months later by a lesion with HPV6 and another lesion co-infected with both HPV6 and HPV32. This indicates that more than one HPV type may be present in MEH lesions in the same patient.

Although MEH is asymptomatic and can regress spontaneously without surgical intervention, diagnosis of MEH is important to ensure differential diagnosis from other benign lesions since these lesions may have overlapping clinical and histologic features. Here I have shown that HPV genotyping complements histologic characterization of benign oral lesions that are suspected of harboring HPV.

2. Premalignant oral lesions

Introduction

In the head and neck, high-risk HPV infection is most often seen in oropharyngeal cancer (OPC), a subset of head and neck cancer that is increasing in incidence worldwide (62). While HPV is strongly associated with OPC, the relationship of HPV to oral squamous cell carcinoma (OSCC), another subgroup of HNC (58), and its premalignant precursor, high-

grade oral epithelial dysplasia (hgOED), is still not clearly defined. While some reports have indicated that HPV is not commonly found in OSCC (73,129); others indicated ~25% prevalence of HPV16/18 in oral dysplasia (130). More recently, a subset of hgOED was shown to be strongly positive for high-risk HPV (131,132). In these studies, the authors indicated that diffuse atypical mitotic-like structures (herein referred to as "mitosoid" cells) and apoptotic cells are unique histologic features warranting the designation "high-risk HPV-associated oral dysplasia"(132) or "HPV-associated Oral Intraepithelial Neoplasia (HPV-OIN)"(131).

In our laboratory, we have also noted hgOED cases that show the widespread mitosoid and apoptotic cells recently described (Group 1 for this study) but in compiling such cases we have also noted that hgOED may also show focal mitosoid and apoptotic cells (Group 2 for this study) while other cases completely lack these features (Group 3). Herein, I explore the association of high-risk HPV in these potential subsets of hgOED with the addition of HPV genotyping. I hypothesize that a high prevalence of high-risk HPV and strong $p16^{INK4a}$ expression will be seen in Group 1 cases with intermediate and lower associations for these variables in Group 2 lesions and Group 3 respectively.

Experimental Procedures

Biopsy sample selection: This study was approved by the IRB at the University of Louisville. Reports of biopsies accessioned into the University of Louisville Oral Pathology Laboratory were searched for keywords and/or phrases that could be associated with HPV infection (i.e. virus, viral, HPV, mitosoid, Bowen, koilocy-, apopto-, multinucleated keratinocyte). All retrieved cases whose diagnosis reported premalignant changes (i.e. dysplasia or carcinoma *in situ*) were then evaluated microscopically for the Group 1 histologic inclusion criteria as previously reported (near full thickness mitosoid and apoptotic cells) (131). Group 2 included cases which had focal mitosoid cells above the basal layer and occasional apoptotic cells (i.e. in at least one or more high power fields but not diffusely through the specimen), suggesting a possible early or isolated infection of those cells with high-risk HPV. Group 3 cases (N=10) lacked these features altogether in the histologic sections studied.

Genomic DNA extraction and HPV detection: The microscopic and clinical information for all cases were blinded before performing HPV genotyping. Genomic DNA extraction and HPV genotyping were performed as described in previously. Type-specific E7 HPV primers were used to confirm the presence of HPV6, 16, 33 and 45.

Immunohistochemistry (IHC) for p16^{INK4a}: Immunohistochemistry for p16^{INK4a} expression in FFPE specimens was performed by the Special Procedures Laboratory (University of Louisville) using an antibody to p16^{INK4a} (CINtec Histology Kit, Roche laboratories). The Leica Bond Polymer Refine Detection (DS9800) system and the Leica BOND III automated IHC stainer were used for histological staining. Staining for p16^{INK4a} was rated according to criteria used by McCord et al (132): grade 2 (strong and diffuse nuclear and cytoplasmic staining of at least the half depth of the epithelial thickness); grade 1 (patchy staining with unstained epithelial cells interspersed among positive cells); and grade 0 (negative, no staining or staining of scattered single cells). This system was modified such that if patchy staining was observed but it included >70% of the dysplastic areas, then it was categorized as grade 2. Also, grade 1 and grade 2 cases were classified as positive for p16^{INK4a} expression, rather than grade 1 cases being "equivocal," and grade 0 cases were categorized as negative.

Statistical analysis: Fisher's exact tests were conducted to evaluate differences between (1) all groups, (2) Group 1 vs. Group 3 lesions, (3) Group 2 vs. Group 3 lesions, and (4) Group 1 vs. Group 2 lesions. As pair-wise intergroup comparisons would produce prohibitively small sample sizes, statistical significance was reported for differences across all 3 groups. As Group 2 and Group 3 were more similar histologically than Group 1, their results were pooled together for some comparisons and are designated as Group 2/Group 3, where applicable. For continuous characteristics, means, standard deviations, and p-values from one-way ANOVA or Student's t-tests comparing groups are reported. To determine if high-risk HPV presence correlated with p16^{INK4a} status (negative vs. positive), stratified exact conditional logistic regression was conducted. Predictor test variables [histologic features alone (Group 1, Group 1 and 2) and in combination with positive p16^{INK4a} expression] of high-risk HPV presence were evaluated for sensitivity, specificity, positive predictive value (PPV) and

negative predictive value (NPV). Statistical analyses were conducted in SAS Version 9.4 (SAS Institute Inc., Cary, NC, USA).

Results

Clinical characteristics of hgOED lesions

The typical clinical appearance for each group consisted of a white or red/white patch or plaque with a variably rough/papillary surface texture which most commonly affected the lateral tongue or floor of the mouth (**Figure 13**). **Table 4** depicts comparisons among all three groups and between Group 1 and Group 2/Group 3. While the lateral tongue and floor of the mouth were most commonly affected in all groups, this location was nearly exclusively involved in Group 2/ Group 3 (95.8%) whereas Group 1 lesions affected other sites in 37.5% of cases (p=0.01). Group 1 lesions were significantly smaller than the Group 2/Group 3 lesions with the latter tending to be >6mm (p=0.03); although, there were many cases for which size data was not available. Patients in Group 1 were typically younger than Group 2/Group 3 patients by 8 years on average (p=0.03).

Histologic features of hgOED lesions

Group 1 hgOED presented with characteristic histologic features as described previously (131,132): abundant mitosoid and apoptotic cells throughout the full thickness of epithelium (**Figure 14**). Group 2, on the other hand, showed only focal apoptotic and mitosoid cells (**Figure 15**). Group 3 showed typical dysplastic features but lacked the combination of mitosoid and apoptotic cells (**Figure 16**).

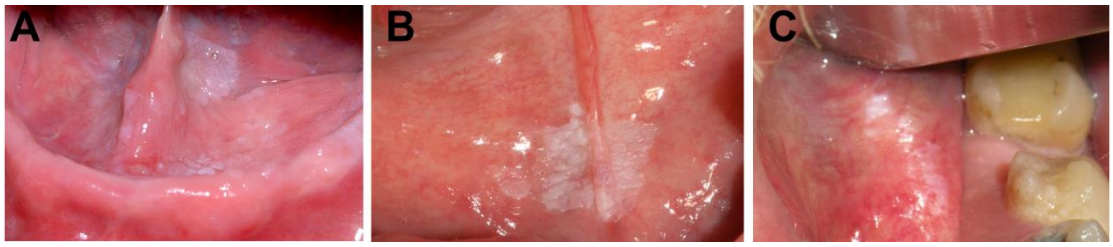

Figure 13: Clinical photograph showing a similar white patch/plaque on the floor of mouth for each group. **A)** Group 1. **B)** Group 2. **C)** Group 3 lesions.

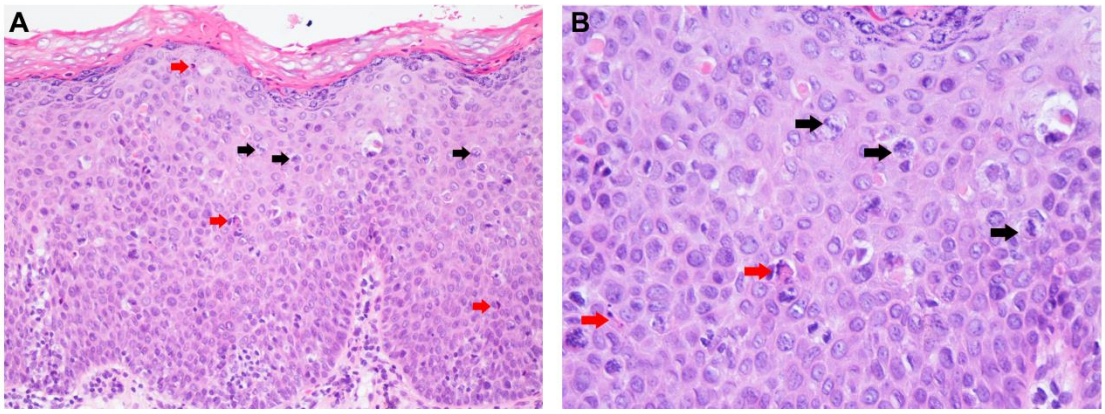

Figure 14: Group 1 hgOED H&E- stained section showing diffuse apoptotic cells (red arrows) and mitosoid cells (black arrows) at **A)** 200x and **B)** 400x magnification.

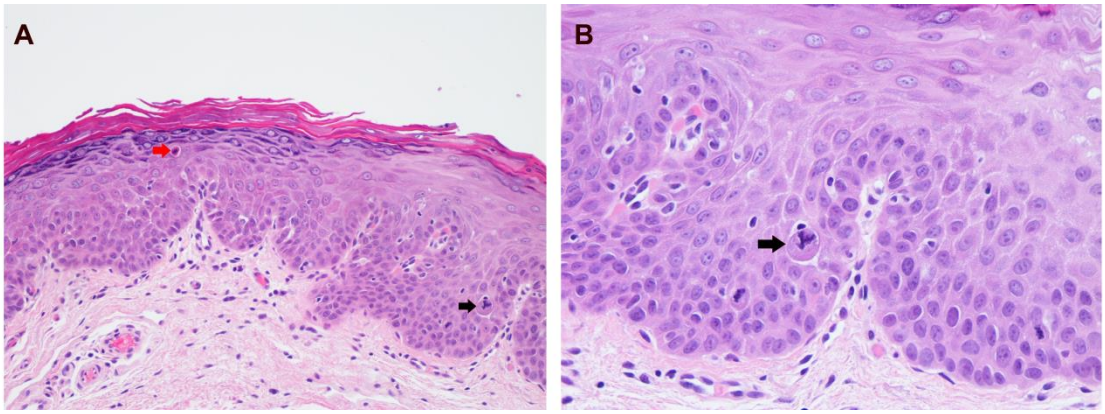

Figure 15: Group 2 hgOED H&E- stained section showing **A)** a focal apoptotic cell (red arrow) and mitosoid cells (black arrows) at 200x, and **B)** at 400x magnification.

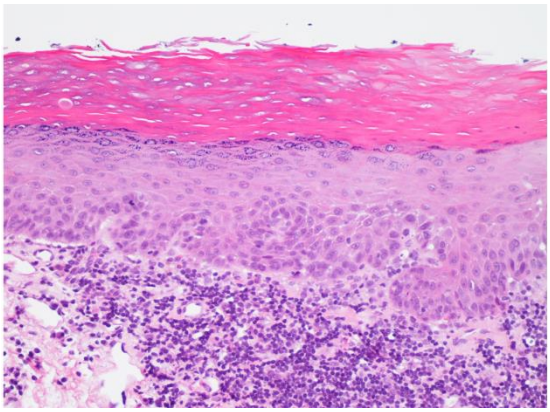

Figure 16: Group 3 hgOED H&E- stained section showing dysplastic epithelial features which lack mitosoid or apoptotic cells at 200x magnification.

HPV status in hgOED lesions

Statistical significant differences among all groups were observed for HPV presence (p=0.02), **Table 4**. High-risk HPV was found significantly more often in Group 1 (81.3%) versus Group 2 (57.1%) and Group 3 (20.0%), (overall p=0.01). HPV16 was seen in all except one HPV-positive case (being HPV33) in Group 1, comprised 7 of 9 (77.7%) of the HPV-positive cases in Group 2 (HPV6 and HPV45 seen in the other 2 cases respectively) and was the only HPV type seen in the Group 3 (2 cases). When Group 2 and Group 3 were combined, Group 1 was significantly more associated with high-risk HPV (p=0.02) and the odd ratio of HPV presence were 5.12 (95% CI 0.99, 33.9) times greater for Group 1 lesions than combined Group 2/Group 3 lesions.

Immunohistochemical (IHC) staining for p16^{INK4a} expression

IHC results for p16^{INK4a} expression between groups are reported in **Table 5**. Strong and diffuse p16^{INK4a} staining (grade 2) was more common in Group 1 cases (75%) than in Group 2 (14.3%) or Group 3 (11.1%), (overall p= 0.0008). Similarly, p16^{INK4a} positive cases (grade 1 and 2, as depicted in **Figure 17**) were significantly higher in Group 1 (93.8%) than in Group 2 (42.9%) or Group 3 (33.3%) (overall p=0.001). The odd ratio of p16^{INK4a} positive status was 23.3 times greater for Group 1 than Group 2/Group 3 (**Table 4**).

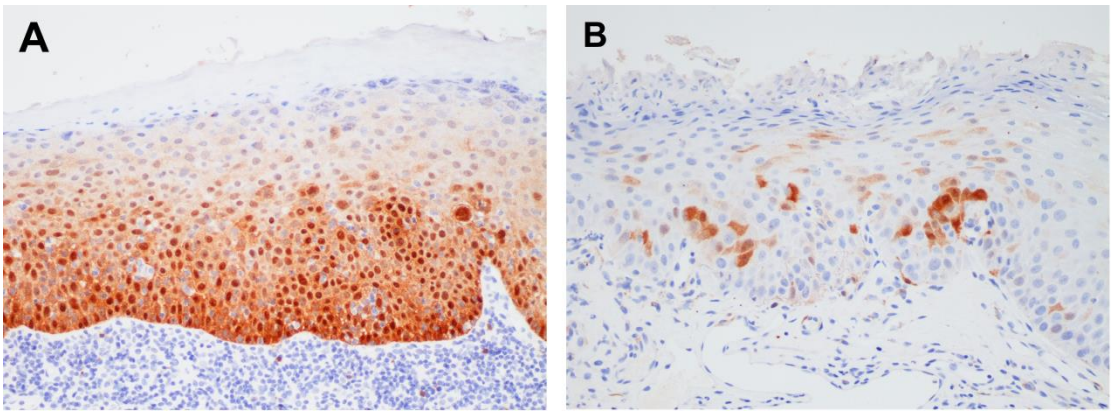

Figure 17: p16^{INK4a} immunohistochemistry. **(A)** Grade 2 (strong and diffuse) staining from Group 1. **(B)** Grade 1 (patchy) staining from the Group 3 group, at 200x magnification.

Association of p16^{INK4a} status with HPV presence

Stratified logistic regression analysis (**Table 5**) showed a significant association between p16 expression and high-risk HPV status (p=0.04). Expression of p16 combined with high-risk HPV positivity was seen frequently in Group 1 (13/16, 81.3%) compared to Group 2 (5/13, 38.5%) and Group 3 (1/9, 11.1%). All high-risk HPV-positive Group 1 and Group 3 samples expressed p16 whereas only 5 of 8 (62.5%) high-risk HPV-positive samples in Group 2 expressed p16^{INK4a}. In Group 2/Group 3 samples, there was a variable association of the presence of high-risk HPV and p16^{INK4a} expression.

Prediction of high-risk HPV presence in hgOED

Analyses of the use of histologic features either alone or combined with p16 p16^{INK4a} expression to predict HPV presence in hgOED are shown in **Table 6**. Histologic features of Group 1 and 2 combined (i.e. mitosoid and apoptotic cell(s) diffusely or focally present) were 91.3% sensitive (95% CI: 72.0%, 99.0%) and 47.1% specific (95% CI: 23.0%, 72.2%) for detecting high-risk HPV with a positive predictive value of 70.0% and negative predictive value of 80.0%. Analysis of Group 1 histologic features alone to predict for the presence of high-risk HPV was more specific at 82.4% (95% CI: 56.7%, 96.2%) but showed a large decrease in sensitivity to 56.5% (95% CI: 34.5%, 76.8%).

The combination of p16^{INK4a} expression and the histologic features of Group 1 lesions were 100% sensitive (95% CI: 75.3%, 100.0%) in predicting the presence of high-risk HPV with a positive predictive value of 86.7% (95% CI: 59.6%, 98.4%), though specificity was only 33% (95% CI: 0.84%, 90.6%), **Table 6**. When Group 1 and 2 were combined then the addition of p16^{INK4a} expression was 81.8% sensitive (95% CI: 59.7%, 94.8%) and 62.5% specific (95% CI: 24.5%, 91.5%) in predicting the presence of high-risk HPV.

Table 4: Clinical variables of hgOED

Variable	Group 1 (N=16) N	%	Group 2 (N=14) N	%	Group 3 (N=10) N	%	p-value Overall	Group1 vs. Group2/Group 3	Odds Ratio Group1 vs. Group2/Group 3 (95% CI)
Gender									
Male	13	81.3	11	78.6	9	90.0	0.88	1.00	0.87 (0.12, 6.93)
Female	3	18.8	3	21.4	1	10.0			
Race									
Black	3	18.8	2	14.3	0	0.0	0.72	0.60	2.63 (0.24, 36.4)
Caucasian	8	50.0	7	50.0	7	70.0			
Unknown[‖]	5	31.3	5	35.7	3	30.0			
Age (median ± IQR)	51.1 ± 10.3		59.0 ± 12.3		59.3 ± 8.5		0.09[†]	0.03[†]	
<40 yrs	1	6.3	1	7.1	0	0.0			0.38 (0.10, 1.30)*
40-55 yrs	10	62.5	4	28.6	3	30.0			
>55 yrs	5	31.3	9	64.3	7	70.0			
Smoking									
Current	7	43.8	5	35.7	4	40.0	0.65	0.21	2.85 (0.46, 21.4) [‡]
Non	3	18.8	6	42.9	5	50.0			
Previous	3	18.8	2	14.3	1	10.0			
Unknown[‖]	3	18.8	1	7.1	0	0.0			
Alcohol									
Current	4	25.0	3	21.4	2	20.0	0.50	0.78	0.80 (0.09, 6.76) [‡]
Non	5	31.3	1	7.1	4	40.0			
Previous	1	6.3	1	7.1	0	0.0			
Unknown[‖]	6	37.5	9	64.3	4	40.0			
HPV									
Negative	3	18.8	5	35.7	8	80.0	0.02	0.03	N/A
Type 6	0	0.0	1	7.1	0	0.0			
Type 16	12	75.0	7	50.0	2	20.0			
Type 33	1	6.3	0	0.0	0	0.0			
Type 45	0	0.0	1	7.1	0	0.0			
HPV risk									
Low-risk HPV	0	0.0	1	7.1	0	0.0	0.01	0.02	5.12 (0.99, 33.9) [§]
High-risk HPV	13	81.3	8	57.1	2	20.0			
Negative	3	18.8	5	35.7	8	80.0			
$p16^{INK4a}$ grading									
grade 2	12	75.0	2	14.3	1	11.1	0.0008	0.0001	7.06 (2.31, 31.2)*
grade 1	3	18.8	4	28.6	2	22.2			
grade 0	1	6.3	8	57.1	6	66.7			
$p16^{INK4a}$ status									
Positive	15	93.8	6	42.9	3	33.3	0.001	0.001	23.3 (2.50, 1,050)
Negative	1	6.3	8	57.1	6	66.7			
Site									
Tongue/ Floor of mouth	10	62.5	13	92.9	10	100.0	0.13	0.01	N/A
Buccal/ labial mucosa	3	18.8	0	0.0	0	0.0			
Soft palate	1	6.3	1	7.1	0	0.0			
Hard palate/ gingiva	2	12.5	0	0.0	0	0.0			

Size									
>6mm	4	25.0	10	71.4	5	50.0	0.09	0.03	0.24 (0.03, 1.14) *,
4-6mm	5	31.3	1	7.1	0	0.0			
<4mm	1	6.3	0	0.0	1	10.0			
Unknown‖	6	37.5	3	21.4	4	40.0			
Duration									
>2 months	4	25.0	5	35.7	4	40.0	0.56	0.36	0.15 (0.00, 2.83)
≤2 months	3	18.8	0	0.0	1	10.0			
Unknown‖	9	56.3	9	64.3	5	50.0			

Abbreviations: IQR, interquartile range; N/A, not applicable; CI, confidence interval
*Odds ratio computed assuming variable is ordinal
† Wilcoxon rank-sum test p-value
‡Current vs. Non; §Positive vs. Negative; ‖ Unknown level omitted for analysis

Table 5: Association between $p16^{INK4a}$ (p16) and high-risk HPV (HR-HPV) in hgOED

	Group 1 (N=16)		**Group 2 (N=13*)**		**Group 3 (N=9)**		**p-value†**
P16 | HR-HPV	**Positive**	**Negative**	**Positive**	**Negative**	**Positive**	**Negative**	
p16 positive	13 (81.25%)	2 (12.5%)	5 (38.46%)	1 (7.69%)	1 (11.1%)	2 (22.2%)	0.04
p16 negative	0 (0.0%)	1 (6.25%)	3 (23.07%)	4 (30.77 %)	0 (0.0%)	6 (66.67%)	

* One low-risk HPV-positive sample omitted for analysis

†Stratified exact conditional logistic regression score test mid-p.

Table 6: Prediction of the presence of high-risk HPV (HR-HPV) in hgOED

Predictor variables for HR-HPV presence	Sensitivity % (95% CI)	Specificity % (95% CI)	PPV % (95% CI)	NPV % (95% CI)
Histologic features alone				
Group 1*	56.5 (34.5, 76.8)	82.4 (56.7, 96.2)	81.3 (54.4, 96.0)	58.3 (36.7, 77.9)
Group 1 and 2†	91.3 (72.0, 99.0)	47.1 (23.0, 72.2)	70.0 (50.6, 85.3)	80.0 (44.4, 97.5)
Histologic features with p16 expression				
Group 1*	100.0 (75.3, 100.0)	33.3 (0.84, 90.6)	86.7 (59.6, 98.4)	100 (2.5, 100.0)
Group 1 and 2†	81.8 (59.7, 94.8)	62.5 (24.5, 91.5)	81.0 (58.1, 94.6)	55.6 (21.2, 86.3)

PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval

*Comparison of Group 1 with Group 2/Group 3

†Comparison of Group 1/Group 2 with Group 3

Discussion

The present study analyzed histologically distinct high-grade oral epithelial dysplastic (hgOED) lesions based on the distribution of mitosoid/apoptotic cells: Group 1 hgOED with diffuse mitosoid cells, Group 2 with focal mitosoid cells and Group 3 hgOED lacking these histologic features. This study is the first study to perform HPV genotyping in histologically distinct hgOED lesions and show significant differences in both the presence of high-risk HPV and $p16^{INK4a}$ expression between these lesions. In previous studies (131,132), researchers employed *in situ* hybridization (ISH) technique using cocktails of low-risk and high-risk HPV probes, which could not identify the specific HPV types as done by PCR-based HPV genotyping.

My data confirm the previous findings (131,132) that high-risk HPV is strongly associated with forms of hgOED with widespread mitosoid and apoptotic cells (Group 1). Though not statistically significant, an intermediate level of high-risk HPV was detected in Group 2 lesions (57.1%) as compared to Group 1 (81.3%) and Group 3 (20%). HPV16 was the most predominant HPV type present in 12/16 (75%) of Group1, 7/14 (50%) of Group 2 and 2/10 (20%) of Group 3. The focal changes in Group 2 cases could be due to early

proliferation of high-risk HPV that could expand throughout the epithelium over time to eventually become widespread as in Group 1 lesions. Correlation of these specific histologic changes in the presence of high-risk HPV with gene and protein expression profiles could help reveal how high-risk HPV infection influences the histologic progression of dysplasia and risk for malignant transformation.

In this study, I showed a significant association between high-risk HPV and $p16^{INK4a}$ expression in Group 1 lesions. Expression of $p16^{INK4a}$ was 100% sensitive for detecting high-risk HPV in Group 1 lesions with a positive predictive value of 86.7%. So, $p16^{INK4a}$ overexpression is a reasonable surrogate marker for high-risk HPV infection in hgOED where widespread mitosoid cells and apoptotic cells are noted. However, 2 cases were $p16^{INK4a}$ positive but negative for high-risk HPV, resulting in only 33.3% specificity (95% CI: 0.84%, 90.6%). Therefore, $p16^{INK4a}$ expression may not be specific for high-risk HPV in hgOED cases that show these unique histologic changes and direct testing for HPV by PCR or *in situ* hybridization (ISH) may be warranted in these instances. My data also show that when these unique histologic features are focal or absent (Group 2 and Group 3 respectively), $p16^{INK4a}$ is not predictive of the presence of high-risk HPV in hgOED.

On combining Group 1 and Group 2 lesions, the sensitivity of these histologic features for detecting high-risk HPV was improved; point estimates for sensitivity increased from 54.2% to 91.7%. However, this was at a cost to specificity—point estimates for specificity decreased from 81.3% to 50.0%. Practically speaking, when a pathologist evaluates a case of hgOED and sees either diffusely distributed or focally dispersed mitosoid and apoptotic cells, there is a high likelihood that high-risk HPV is present and warrants direct testing for HPV. Whether focal changes seen in Group 2 lesions are a precursor to more widespread changes observed in Group 1 lesions requires further study.

While a causative role of high-risk HPV is clearly implicated in OPC, its role in OSCC is still largely unknown (74) and even less is understood about its role in the development of pre-malignancy and subsequent malignant transformation. An initial challenge to studying HPV-associated disease progression is to identify high-risk HPV infection in the premalignant state. For OPC, tumorigenesis often occurs deep within the crypts without a surface

premalignant precursor (47) whereas OSCC often develops from a clinically apparent lesion (84) with microscopic evidence of dysplasia. Also, there is no reason to clinically evaluate the tonsillar crypts unless there are symptoms (e.g. neck mass, sore throat), which do not occur until cancer has already developed. In contrast, routine oral examination (as performed in the dental care) allows detection of early oral premalignant lesions thus providing the unique opportunity to study how HPV infection may initiate dysplastic changes or propel them forward to malignancy. While the mechanisms of HPV-driven neoplasia in the oropharynx compared to the oral cavity likely differ, the study of these oral cavity lesions may be the best *in vivo* opportunity to study the evolution of head and neck HPV-associated neoplasia.

3. Carcinoma of the head and neck

Introduction

Head and neck cancers are malignant tumors that develop in the upper aerodigestive epithelium. A subset of head and neck cancers (HNCs), mostly oropharyngeal cancer, has been found to be strongly associated with HPV infection, particularly high-risk HPV types 16 and 18. Other risk factors for HNC are tobacco and alcohol use (61). Although the prevalence of smoking is decreasing in the United States, there has been a recent increase in the incidence of HPV-positive oropharyngeal cancers (62). About 72% of oropharyngeal cancers are caused by HPV in the United States (62). It is estimated that around 2,400 and 9,400 new cases of HPV-associated oropharyngeal cancers are diagnosed in women and men, respectively, each year in the US. HPV-associated oropharyngeal cancers now outnumber the cases of these cancers attributable solely to smoking or alcohol use (133).

Immunohistochemical analysis (IHC) for $p16^{INK4a}$ expression is frequently used to determine HPV status in the clinic, but many reports have shown that $p16^{INK4a}$ is not always a good surrogate for HPV infection (18,46,48). This study assessed the presence of HPV by using the PCR-based method to detect HPV DNA and compared the HPV-positive status with cancer anatomic site and $p16^{INK4a}$ expression.

Experimental Procedures

Tissue Samples: This study was approved by the IRB at the University of Louisville. Patients who attended the Head and Neck multidisciplinary clinic for evaluation of their cancer were asked to provide written consent. HNC specimens were classified into one of five categories according to anatomical subsites: **a.** Oral cavity (includes lip, anterior tongue, gum, floor of mouth, hard palate); **b.** Oropharynx (includes the base of tongue, lingual tonsil, soft palate, uvula, tonsil, and oropharynx); **c.** Hypopharynx (includes pyriform sinus, hypopharynx); **d.** Larynx (includes glottis, supraglottis, subglottis); and **e.** Other unspecified cases (includes sites which were not within one of the categories listed above and those of unknown primary cancer). This categorization is based on The International Classification of Diseases (134), and the method used by Hashibe *et. al* (135). The clinical stage of each cancer was determined using the TNM staging system, 7th edition (136). Demographic and disease characteristics of patients were collected from the deidentified database and researchers were blinded to the HPV status of the patients before serological analysis.

Genomic DNA extraction and HPV detection**:** DNA was extracted from fresh frozen or formalin-fixed, paraffin-embedded (FFPE) specimens using DNAeasy Blood & Tissue kit (Qiagen, USA) as per manufacturer's instructions with RNAase (20µl of 20 mg/ml) treatment. FFPE samples were deparaffinized using xylene and washed before DNA extraction. HPV detection and genotyping were performed, as described previously. β-globin amplification was done used for an internal control.

$p16^{INK4a}$ ***immunohistochemistry: I***mmunostaining was performed using standard protocols in the UofL pathological laboratory. IHC for $p16^{INK4a}$ was considered positive if there was strong and diffuse staining present in >70% of the malignant cells. The results of the $p16^{INK4a}$ IHC for malignant specimens were provided by the Cancer Database and Specimen Repository (CDSR) at the James Graham Brown Cancer Center, University of Louisville. There were cases in which $p16^{INK4a}$ IHC was not performed as part of a patient's standard diagnostic workup and hence were unavailable at the time of analysis.

Statistical analysis: Distributions of discrete lesion characteristics were summarized by frequency and percentage. Fisher's exact tests or Students t-tests were conducted to

evaluate differences between variables using SigmaPlot Version 12.5 (Systat Software, Inc., San Jose California, USA).

Results

Demographic Information of HNC Patients

Summary on the distribution of 54 HNC specimens is shown in **Table 7**. Age ranged from 35-87 years and 81.5% of patients were males with two exceptions. HPV-positive HNC patients were about 6 years younger than patients whose tumors were HPV-negative ($p<0.05$). No significant difference in cancer stages was found between HPV-positive and HPV-negative HNC.

HPV-positive HNC patients

HPV DNA was detected in 18 out of 54 HNC specimens (**Table 7**). When the MY and GP amplicons (**Figure 18**) were sequenced, 16 were genotyped as HPV16 and other two were HPV18 and HPV33. Results were further verified using HPV type-specific primers. Of note, there were many samples, which produced a band around the size of MY amplicon, but were not detected to have HPV DNA after sequencing.

Correlation of HPV positivity with HNC anatomic sites and p16^{INK4a} status

HPV-positive cases were significantly associated with oropharyngeal cancer (**Table 7**), $p<0.001$. In this study, only 16.7% of total HPV-positive patients had oral cavity cancers. Positive HPV status was also significantly correlated with positive p16^{INK4a} IHC staining ($p<0.001$).

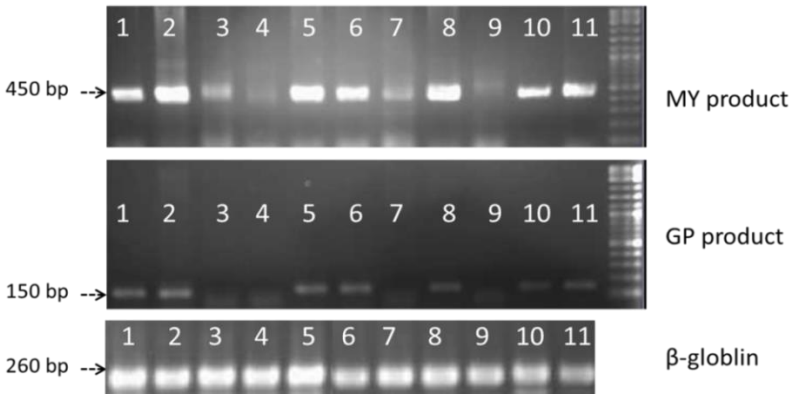

Figure 18: HPV DNA detection in HNC samples by PCR using MY09/11 and GP5+/GP6+ consensus primers. β-globin was used as the loading control.

Table 7: Demographics of HPV positive and HPV negative HNC patients

Category	**HPV DNA positive (N=18)**		**HPV DNA negative (N=36)**		**p-value**
	n	%	n	%	
Gender					
Male	16	88.9	28	77.7	NS
Female	2	11.1	8	22.2	
Age (years)	55.11± 8.4		61.1 ± 10.8		0.022*
Anatomic site					
Oropharynx	11	61.1	3	8.3	<0.001*
Oral cavity	3	16.7	11	30.6	
Others (Hypopharynx, larynx)	4	22.2	22	61.1	
Stage					
I	0	0.0	1	2.8	NS
II	1	5.6	12	33.3	
III	4	22.2	13	36.1	
IVA	9	50.0	4	11.1	
IVB	0	0.0	0	0.0	
IVC	0	0.0	0	0.0	
Unknown†	4	22.2	6	16.7	
$p16^{INK4a}$ status					
Positive	13	72.2	3	8.3	<0.001*
Negative	0	0	10	27.8	
Unknown†	5	35.7	23	63.9	

*Significant values, NS: non-significant

†Unknown values omitted during analysis

Discussion

In this study, an initial characterization of the malignant specimens was done and the obtained data was used for their further analysis in subsequent chapters. Malignant tissue samples from different histological sites of the head and neck (oropharynx, oral cavity, hypopharynx, and larynx) were examined. The median age of HPV-positive HNC patients was significantly younger than HPV-negative patients. A similar trend is seen in HPV-positive OPC and OSCC patients in younger patients compared to those with HPV-negative cancers (62,137). When cases were stratified according to location, the data showed that oropharyngeal sites (tonsils, posterior tongue, and oropharynx) were the most common sites affected in patients who were HPV-positive. My results were consistent with the increasing incidence of HPV infection in oropharyngeal cancers (60%-100%) (62,133,138,139). HPV-negative cancers were mainly from non-oropharyngeal cancers of the head and neck region. Further, the data showing only 16.7% (3/18) of HPV-positive OSCC samples supports the findings of previous studies that HPV prevalence is lower in OSCC (13-47%) (58,140,141). As expected, HPV type 16 was the predominant type found in our specimens, which was also reported in the majority of other studies (69,82,138,142). Other high-risk types- HPV18 and HPV33 were also observed. While HPV16 is the most common type detected in OPC, HPV33 and HPV18 have shown to be rare but significant factors in OPC (32,143,144).

Results indicated that HPV-positive cancers are significantly associated with positive $p16^{INK4a}$ expression, confirming the finding of previous reports, particularly in OPCs (48). Studies also suggest that factors/mechanisms other than HPV infection likely stimulate $p16^{INK4a}$ expression, mainly in OSCC (46,74,76). 72.2% of the HPV-positive cancers showed positive $p16^{INK4a}$ staining, though most could not be evaluated for $p16^{INK4a}$ status. The majority of HPV-negative OSCC was $p16^{INK4a}$ negative. Out of 3 HPV-positive OSCC, two showed positive $p16^{INK4a}$ staining (the other could not be determined). These data did not reveal any obvious results towards positive or negative $p16^{INK4a}$ status in OPC and OSCC because of small sample size and limited available data.

In summary, my study is in agreement with previous results showing a clear association of high-risk HPV with HNCs, particularly oropharyngeal cancers. In addition to

HPV genotyping and $p16^{INK4a}$ staining, characterization of mitosoid cells would be useful. In earlier sections of this chapter, I have shown that mitosoid cells are strong biomarkers of HPV infection in benign MEH and distinct subset of pre-malignant oral tumors. It was not possible to characterize mitosoid cells in malignant specimens due to unavailability of histological slides. Further studies characterizing mitosoid cells in cancerous tissues of the head and neck would be useful to determine whether these histological features are consistently present during premalignant to malignant transformation. In addition to histologic biomarkers, identification of HPV-associated serum biomarker would be non-invasive and early method to diagnose HPV etiology in HNCs (discussed in Chapter III). Also, detailed molecular studies are needed to determine mechanism how HPV influences malignant transformation in HNCs (discussed in Chapters IV and V).

CHAPTER III

POTENTIAL SERUM BIOMARKER FOR DETECTION OF HPV-ASSOCIATED HEAD AND NECK MALIGNANCY

Chapter Overview

HPV-related head and neck cancer incidence is increasing rapidly worldwide including the United States, but there are no standard non-invasive ways to detect HPV in these cancers. Although $p16^{INK4a}$ overexpression in tumor tissues is widely accepted as a surrogate marker for HPV positivity, studies reported that non-HPV cancers also express $p16^{INK4a}$. In chapter II, I have shown that mitosoid cells could serve as a strong histologic biomarker of HPV infection in the head and neck tumors. Detection of both $p16^{INK4a}$ expression and mitosoid cells require cancer tissues specimens. Therefore, there is a need to identify serum biomarker of HPV infection that could carry the potential of earlier and non-invasive methods of viral detection. Therefore, in this chapter, I determined whether titers for antibody to the HPV E7 protein could also be used as a suitable biomarker for head and neck malignancies associated with HPV. To this end, HPV E7 antibody titer was analyzed together with $p16^{INK4a}$ staining and HPV genotyping in tumor tissues to access their potential uses as biomarkers for the presence of HPV. My data represent the first report that the combination of E7 serology and $p16^{INK4a}$ staining represents a strong diagnostic marker for underlying HPV etiology in HNCs (145).

Introduction

Evidence of an etiologic role for HPV in head and neck squamous cell carcinoma has been accumulating, with high-risk HPV16 (and to a lesser extent HPV18) detected in carcinomas in this region. The $p16^{INK4a}$ protein is overexpressed in HPV-positive tumors as a result of the HPV oncoprotein E7 inactivating the tumor suppressor pRb protein. This inactivation leads to increased expression of downstream genes like $p16^{INK4a}$ normally

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repressed by pRb. HPV DNA detection (by *in situ* hybridization or PCR) and $p16^{INK4a}$ immunohistochemistry are currently used to determine HPV status. Both of these tests require cancer tissues samples. Novel diagnostic techniques using serum samples could carry the potential of earlier and non-invasive methods of detection.

Studies have been done to evaluate serologic markers of HPV infection in HNC. HPV serology studies have focused on detecting HPV16 antibodies and have focused mostly on the L1 protein (146-149), with few studies involving E6 and E7 oncoproteins (149-152) or E1/E2 proteins (152). Antibodies to HPV16 E6 and/or E7 are suggested to be more specific for HNC than L1 antibodies (149,150). The presence of HPV L1 and E2 antibodies may indicate benign infection whereas E6 and E7 antibodies are more specific for premalignant and malignant lesions (*i.e.* transcriptionally active HPV infection). Antibodies against E6 and E7 proteins are largely HPV type specific. HPV18 DNA has been also detected in HNCs but only a few studies have examined the seroprevalence of HPV18 in HNCs (153,154). Some studies have suggested a correlation between HPV16 E6/E7 seropositivity and clinical outcome of HNC patients (155-157).

My study examined the presence of HPV16 and 18 specific antibodies to E7 oncoprotein in sera of HNC patients and compared the seropositive status with anatomic site of cancers, $p16^{INK4a}$ IHC positivity, HPV tumor DNA, smoking status and treatment outcome. Currently, there are no standard commercially available serologic tests that detect HPV-antibodies against any HPV proteins. Although HPV serological studies have been reported; most of them have focused on HPV16 antibodies (not HPV18) and fewer have conducted serology both before and after treatment of HNC patients.

Experimental Procedures

Serum samples: This study was approved by the IRB at the University of Louisville. Blood was collected from pathologically confirmed HNC patients during the period from 08/27/2009 to 07/30/2014. Blood samples were kept at room temperature for 30 minutes to clot and then were centrifuged at 3,000 rpm for 10 min to obtain serum. Serum aliquots were obtained from the Cancer Database and Specimen Repository (CDSR) at the James Graham Brown Cancer

Center (JGBCC). I analyzed 92 de-identified serum samples (including 17 follow-up sera) from 75 patients. HNC was classified into anatomical subsets, as described previously in chapter II. Sera from two cervical cancer patients who were seropositive for HPV16 or HPV18 were used as positive Group 3.

***E7 proteins of HPV16 and 18*:** Previously, E7 oncoproteins of HPV16 and 18, fused with MBP (maltose-binding protein), were expressed in *E.coli* and purified on amylose column (158). These fusion proteins were employed as antigens for ELISA in this study.

Serological Analysis by ELISA: Total IgG antibody against E7 oncoproteins of HPV16 and HPV18 were examined by direct ELISA using recombinant E7 proteins as antigens. Briefly, 500ng protein/ well in 100µl of 50mM bicarbonate buffer (pH 9.6) was coated onto Immulon™ ELISA microplates (Thermo Scientific, USA) using an overnight incubation at 4°C. After three washings with 200µl of PBS, wells were blocked with 100µl of 5% PBS-A (PBS containing 5% bovine serum albumin) and incubated for 1 h at 37°C. Wells were then washed three times with 200µl of PBS. Sera diluted to 1/100 in 1% PBS-A was added for 1 h at 37°C followed by the alkaline-phosphatase-(AP-) conjugated goat anti-IgG of human at 1/1,000 dilution in 1% PBS-A for 1 h at 37°C. After the addition of 100µl of the AP-chromogenic substrate (Sigma-104 p-nitrophenyl phosphate substrate; Sigma, St Louis, MO), the absorbance was measured at 405 nm using Gen5 Microplate Reader and Imager Software in Synergy™ HT Multi-Mode Microplate Reader (BioTek Instruments, Inc., VT, USA).

All serum samples were analyzed at the same time to avoid experimental variations. ELISA for all samples was performed at least three times. Human sera, which had been previously tested as positive for HPV16 or HPV18 E7 oncoproteins, were used as positive controls for each experiment. Wells without antigen or serum was used as negative control and wells containing the only substrate were used as background control.

HPV DNA detection in HNC tissues: FFPE tumor samples with corresponding serum specimens were available HPV DNA detection and genotyping in 11 of the HNC cases. DNA was extracted and HPV genotyping was performed, as described previously in chapter II. HPV DNA status of the tumor tissues was then correlated with HPV E7 seropositivity.

p16^{INK4a} immunohistochemistry (IHC): Immunostaining for p16^{INK4a} results were obtained from a deidentified database of the Cancer Database and Specimen Repository (CDSR) at the James Graham Brown Cancer Center. IHC for p16^{INK4a} expression was considered positive if there was strong and diffuse staining present in >70% of the malignant cells. p16^{INK4a} result was then correlated with HPV E7 seropositivity.

Statistical Analysis*:* The seropositive and seronegative groups were determined by two different methods regarding the distribution of absorbance values. In the first method, seropositivity was defined as samples whose absorbance values were 2 standard deviations (SD) from the mean value. We noticed that there was no difference in demographics for HPV seropositivity for both 2 and 3 SD of the mean. In the second method, absorbance values were observed based on quintile plots and two different slopes on the plots were determined using the non-linear procedure (158). Thresholds of the seropositive group were determined by the cross points of two slopes on the plots, and the samples whose absorbance values were greater than or equal to the threshold values. Both methods used to define seropositivity were in agreement. The ELISA values greater than the cut-off (1.04 and 0.905 for HPV16 E7 and HPV18 E7, respectively) were considered seropositive. Once the seropositive group was determined, the associations were examined between the seropositive group and all available variables using Chi-square test for discrete variables and independent t-test for continuous variables at p=0.05. All data analyses were conducted by SAS 9.3 and Microsoft Office Excel.

Results

Demographic Information of HNC Patients

Summary on the distribution of 75 HNC and 25 seropositive patients is shown in **Table 8**. All groups (total, seropositive and seronegative) were the average age of 57 years. None of the seropositive cases were less than 40 years.

HPV E7 Seropositivity of HNC Patients

Antibodies against E7 oncoproteins of HPV16 and 18 were examined by ELISA in 92 serum samples, including 17 follow-up samples (**Figure 19**). Of the 75 patients, 14 and 8 patients were seropositive for the oncogenic E7 proteins of HPV16 and HPV18, respectively.

Three patients were cross-reactive for E7s of both HPV types. Four follow-up sera were seropositive (2 for HPV16 E7, 1 for HPV18 E7 and 1 was cross-reactive for both HPV types) (**Figure 19**).

Correlation of E7 Seropositivity with HNC anatomic sites and p16^{INK4a} status

I evaluated the association between serology and HNC anatomic sites. Seropositive cases were strongly correlated with oropharyngeal cancer (**Table 8**). Positive HPV serology was also significantly associated with positive p16^{INK4a} IHC (**Table 8**). I further compared HPV serology with p16^{INK4a} IHC in oropharyngeal cancer as compared to other anatomic sites of the head and neck (**Table 9**). Only patients whose p16^{INK4a} IHC results were known were included, excluding unspecified cases. The association between E7 serology and p16^{INK4a} IHC was significant in oropharyngeal carcinoma as well as in other HNC sites (**Table 9**). Although the sensitivity of E7 serology as a diagnostic marker in HPV-driven HNC (as defined by positive p16^{INK4a} IHC) was only 62.5%, E7 serology showed a high specificity and positive predictive value (94.7% and 95.2% respectively) (**Table 10**). Isolating only oropharyngeal cancers, where the role of HPV is much more clearly defined, the positive predictive value was 100%.

Table 8: Demographics of 75 HNC and 25 HPV E7 seropositive patients

Category	**Total HNC patients** (N=75)	**Seropositive** (N=25)	**Seronegative** (N=50)	**p-value**
Gender				0.186
Male	67 (89.33%)	24 (96%)	43	
Female	8 (10.67%)	1 (4%)	7	
Age	57 ± 10	57.56 ± 7.75	56.60 ± 11.12	0.7002
< 40 yrs	4	0	4	
40 - 59 yrs	43	16	27	
≥ 60 yrs	28	9	19	
Anatomical sites				0.0006*
Oropharynx	39 (52%)	20 (80%)	19	
Others (Oral cavity, Hypopharynx, Larynx)	36 (48%)	5 (20%)	31	
Stage				0.674
II	4	1	3	
III	15	7	8	
IVA	48	15	33	
IVB	2	1	1	
IVC	3	0	3	
Undetermined	3	1	2	
$p16^{INK4a}$ status				<0.001*
Positive	32 (42.1%)	20 (80%)	12	
Negative	19 (25.33%)	1 (4%)	18	
Unspecified†	24 (31.57%)	4‡ (16%)	20	
Smoking status				0.3556
Past or current smokers (>10 pack-years)	55(73.33%)	20 (80%)	35	
Never-smokers	20 (26.67%)	5 (20%)	15	
Treatment response				0.45
Complete response	33	13	20	
Partial response	10	4	6	
Progression	11	2	9	
Not assessed	21	6	15	

*Significant values

†Unspecified $p16^{INK4a}$ status was not considered during statistical analysis

‡$p16^{INK4a}$ IHC results were unavailable for 4 seropositive cases.

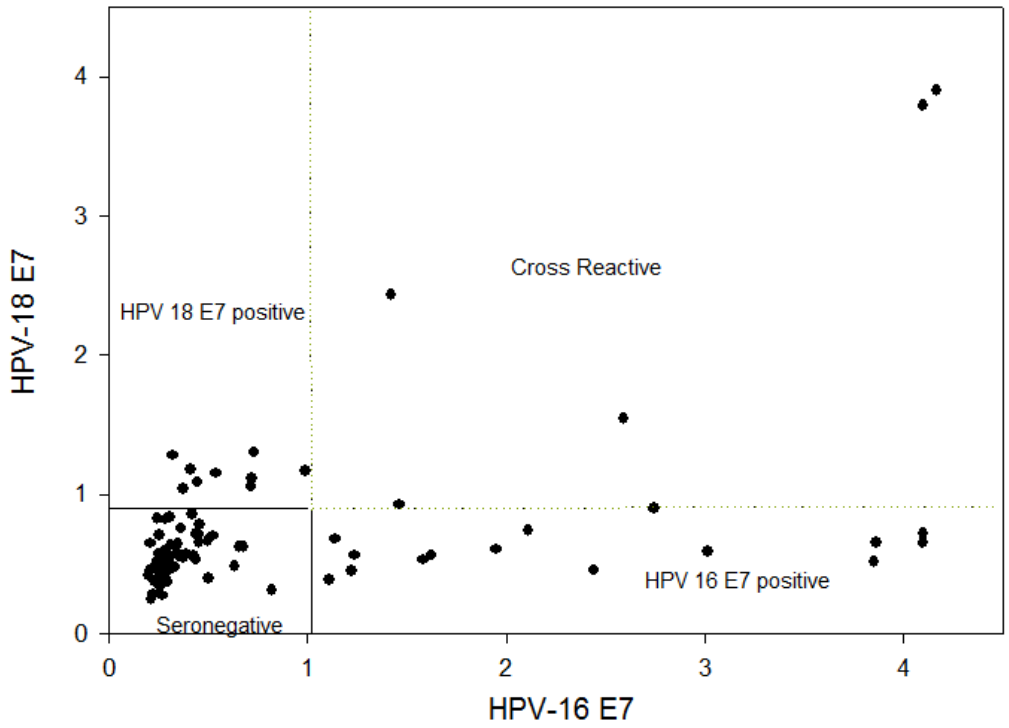

Figure 19: Scatter plot of HPV16 and HPV18 E7 antibody titers of 92 serum samples from 75 HNC patients. Cut-off lines were drawn at 1.04 for HPV16 E7 and 0.905 for HPV18 E7. The values that were higher than the cut-off were considered seropositive.

Table 9: E7 serology and $p16^{INK4a}$ status in oropharyngeal and other HNCs

Site	**E7 serology** (HPV16, HPV18 or both)	**$p16^{INK4a}$ status**		**p-value***
		Positive	**Negative**	
Oropharynx (N=34)	Seropositive (N=17)	17	0	0.0030
	Seronegative (N=17)	10	7	
	Total	27	7	
Others (N=17)	Seropositive (N=4)	3	1	0.0221
	Seronegative (N=13)	2	11	
	Total	5	12	

*Unspecified $p16^{INK4a}$ status was not considered during statistical analysis

Table 10: Diagnostic measures based on E7 serology and $p16^{INK4a}$ status

Site	Sensitivity (%)	Specificity (%)	PPV* (%)	NPV* (%)
Total (n=51)	62.5 (20/32)	94.7 (18/19)	95.2	60
Oropharynx (n=34)	63	100	100	41.2
Others (n=17)	60	91.7	75	84.6

* PPV and NPV denote positive predictive value and negative predictive value respectively

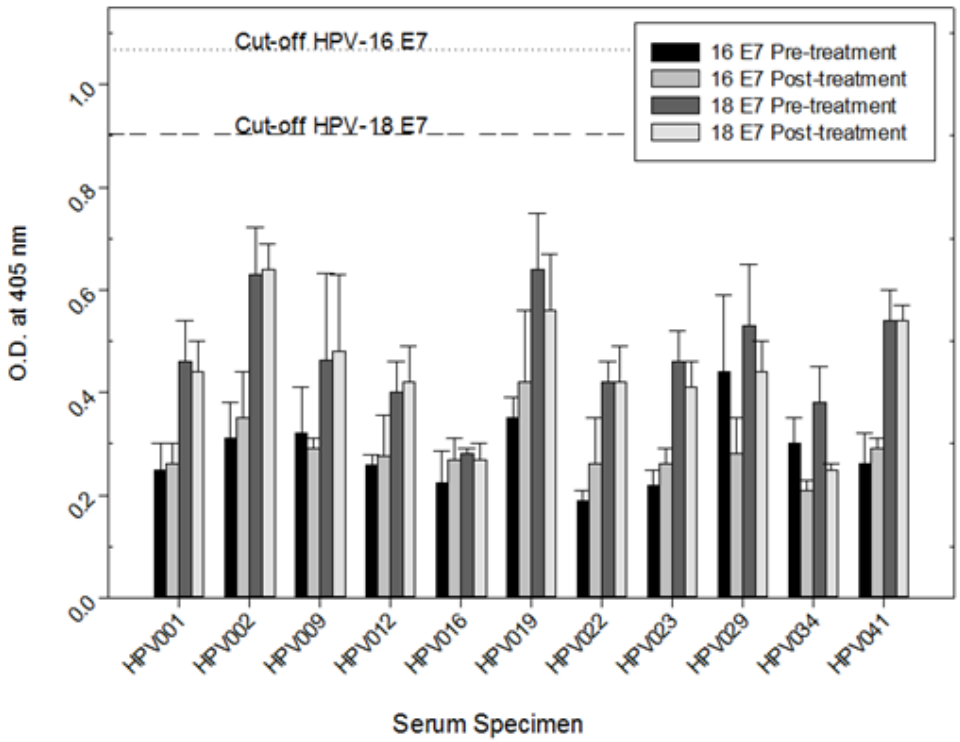

Figure 20: HNC patients (N=11) showing negative HPV16 and HPV18 E7 antibody titers both before and after treatment.

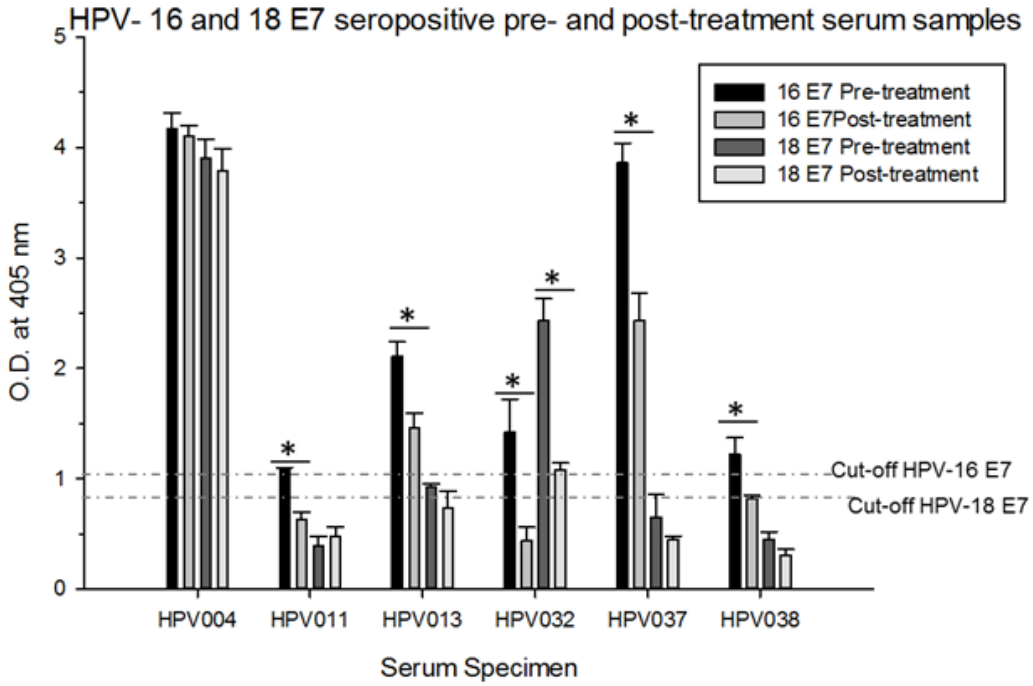

Figure 21: HNC patients who showed positive antibody titers before treatment revealed a significant decrease in titer values after treatment, except in one case (HPV004). Five of patients who showed decrease in antibody titer had complete/partial response to treatment

Correlation of E7 Seropositivity with treatment response

Seventeen follow-up serum samples were available for analysis. Sera (N=11) that were seronegative before treatment, also revealed negative serology after treatment (**Figure 20**). I evaluated antibody status of the remaining 6 follow-up samples and compared with respective seropositive pretreatment samples (**Figure 21**). Follow-up samples were collected at a median of 96 days post-treatment (range 87-170 days). Treatment response at this time point was supplied by the biorepository database and was determined clinically and in some cases, radiographically, when imaging was available. Post-treatment serum antibody titer against HPV16 and 18 E7 decreased significantly compared to those at pretreatment ($p < 0.01$) in 5 patients, who had a complete or partial response to treatment. One case (HPV004) whose titer was decreased, but not significantly, also showed a complete response to treatment. Three patients (HPV011, HPV032, and HPV038) became seronegative during follow-up (**Figure 21**).

Correlation of E7 Seropositivity with HPV DNA

Eleven HNC tissues were available for HPV detection and genotyping (**Figure 22**). When the MY and GP amplicons of these seven samples were sequenced, 6 were genotyped as HPV16 and 1 sample as HPV33. A sample (HN027) showing HPV33 was confirmed by another PCR using HPV type 33- specific primers against oncogene E7. One sample (HN021), which produced a band around the size of MY amplicon, was not detected to have HPV DNA after sequencing. Samples (HN013, HN019, and HN025), which did not produce bands consistent with MY or GP, were negative for HPV DNA. Altogether, HPV DNA was detected in 7 samples out of 11 HNC tissues. Positive HPV DNA in cancer tissue was mainly from the oropharynx.

I correlated HPV DNA status with the HPV serology of the patients whose tissues were available for DNA extraction and HPV genotyping. The association of HPV16 E7 serology was strong among the samples tested positive for HPV16 DNA (N=6), except in one case. This exceptional case (HN014) was seronegative in repeated assays even though it was HPV16-DNA positive. I verified that detected HPV DNA was not a result of sample contamination. There were two cases (HN013 and HN021) in which HPV-DNA was not detected in their tissue, but sera were positive for HPV-16 E7. The reason behind this might be due to multiple HPV infections in a single patient, that makes MY/GP- PCR method inefficient and subsequent typing by sequencing is fraught with difficulties in revealing multiple types. The sample (HN027), which was positive for HPV33 DNA, was HPV18 E7 seropositive, which might be due to the cross-reactivity between epitopes of HPV33 E7 and 18 E7 proteins. A sample (HN025) was negative both for HPV DNA and E7 serology. In overall, the sera of 86% of HPV DNA-positive patients (6 of 7) gave positive ELISA results.

Correlation of E7 Seropositivity with smoking status

I assessed the association of seropositivity with smoking status. I defined “smokers” as the past and current smokers who smoked 10 or more pack-years. 73% of the total HNC patients were smokers, indicating the high smoking prevalence in our population, regardless of HPV positivity. Also, seropositivity did not significantly correlate with smoking status ($p > 0.05$), **Table 8**. Only 20% (5 out of 25) seropositive cases were never smokers.

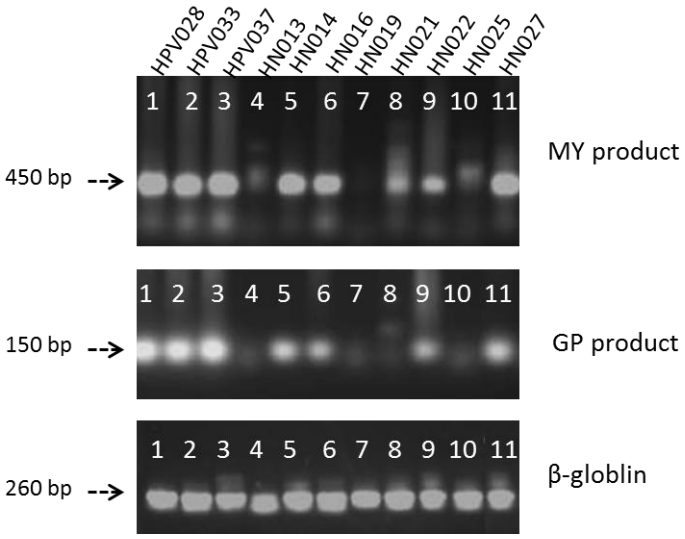

Figure 22: HPV–DNA detection in HNC tissue samples using MY and GP consensus primers. β-globin was used as the internal control.

Discussion

This study evaluated serological biomarkers for clinical assessment of the role of HPV in HNC. Although studies have been done to detect HPV L1 (VLPs) (146-149) and E1/ E2 antibodies (152), these antibodies mostly indicate early benign HPV infection or vaccination. Since cancer usually develops after years of persistent HPV infection; detection of antibodies for E6/E7 oncoproteins may be helpful in the serological diagnosis of malignant lesions.

Serial monitoring of HPV serology may also be useful in predicting a patient's response to treatment. The decline of anti-HPV16 E6 and/or E7 antibodies after treatment has been shown in cervical cancers (159-161) and with a few studies in HNC (156,157). From our laboratory, Drs. Jenson and Ghim had followed cervical cancer patients with E7 serological tests and found profound IgG response against E7, which was associated with tumor stability or dormancy (162). In a small subset of patients, I observed a significant decline in HPV E7 antibodies after treatment, except for one patient. I also observed that sera that were seronegative before treatment remained so after treatment of patients. In addition, some studies have suggested that HPV16 E6/E7 seropositivity is prognostic of a favorable clinical outcome of HNC patients (156,163). HPV status as defined by positive E6/E7 serology

and positive tumor HPV status (HPV DNA and $p16^{INK4a}$) is suggested to correlate even more strongly with favorable prognosis than tumor HPV status (HPV DNA and $p16^{INK4a}$ IHC) alone (163).

When seropositive cases were examined, I observed a statistically significant correlation with oropharyngeal cancer, but not with other anatomic sites of HNC. My results were consistent with the increasing incidence of HPV infection in oropharyngeal cancers. I demonstrated that HPV serology correlates well with routine methods of testing of HPV status (HPV DNA detection and $p16^{INK4a}$ IHC) as 86% (6 of 7) of HPV DNA positive HNC patients also displayed positive serology. Further, E7 antibody titer in patients' sera was strongly correlated with $p16^{INK4a}$ positivity in HNC tissue samples. Also, among HNC samples that tested positive for HPV16 DNA, the association of HPV16 E7 serology was strong.

In the present study, HNC patients were of the average age of 57 years, regardless of HPV positivity. This population of patients demonstrated a high prevalence of tobacco use, with 74% of patients with $p16^{INK4a}$ -positive HNC reporting >10 pack-years. Previous studies have suggested that this intermediate risk group has inferior survival compared to non-smokers with HPV-induced HNC. HPV E7 seropositivity did not correlate with smoking status in our study population, possibly secondary to the strikingly high smoking prevalence. Indeed, some cases of HNC may be caused by a combination of tobacco and HPV (164).

In this study, I also found that E7 antibody levels declined with response to treatment of a patient, though post-treatment serum was available in a limited subset of patients. This study was done with only a single follow-up sample per patient. Therefore, further studies in a larger sample size, including longitudinal follow-up of patients could help to identify if serial monitoring of E7 serology may be useful in predicting a patient's respond to treatment.

In summary, HPV-specific E7 antibody levels in the sera of patients with oropharyngeal cancer are complementary to the well-established HPV histological detection methods. Given the high positive predictive value of E7 serology for $p16^{INK4a}$ positivity, particularly in cancers of the oropharynx, HPV serology may be particularly useful in cases when insufficient tissue is available to carry out $p16^{INK4a}$ immunostaining for detection of HPV-associated disease. Further comparisons between potential tissue and serum biomarkers *i.e.*

expression of $p16^{INK4a}$, presence of mitosoid cells and positive E7 serology, would be useful to identify the best HPV-associated biomarker in head and neck tumors.

CHAPTER IV

HPV DNA INTEGRATION AND VIRAL GENE METHYLATION DURING HEAD AND NECK CANCER PROGRESSION

Chapter Overview

In order to better understand the progression of HPV infection in premalignant lesions to head and neck cancers, I aimed to characterize HPV-associated genetic and epigenetic alterations involved in head and neck carcinogenesis. Studies in cervical cancer indicate that HPV-linked malignant conversion is associated with the specific molecular events *i.e.* HPV integration and HPV methylation. In contrast, little is known regarding malignant progression in HPV-linked head and neck cancers. Therefore, frequencies of HPV DNA integration and methylation were profiled in the premalignant and malignant head and neck tumors and underlying mechanisms associated with head and neck carcinogenesis were studied.

Introduction

Head and neck cancer (HNC) comprises squamous cell carcinoma (SCC) of the oral cavity (oral squamous cell carcinoma, OSCC), oropharynx (oropharyngeal squamous cell carcinoma, OPC), hypopharynx and larynx. In addition to excessive tobacco and alcohol consumption, the human papillomavirus (HPV) infection has been established as an etiological factor for HNCs, particularly OPC. Patients with HPV-associated cancers display better treatment response and survival than patients whose tumors are HPV-negative, independent of treatment strategy (63,64). While HPV is strongly associated with OPC, the relationship of HPV to OSCC and its premalignant precursor (high-grade oral epithelial dysplasia, hgOED) are not clearly defined. Our group (127) and few others (131,132) have

recently reported that HPV is strongly associated with a certain histologic subset of hgOED. In this chapter, I aimed to further characterize these premalignant lesions and their relationship with malignant lesions to better understand the underlying molecular mechanisms in HPV-associated head and neck carcinogenesis.

The major viral oncogenes *E6* and *E7* are generally thought to be responsible for HPV-associated malignancy, as shown in **Figure 2**. Their expression is mainly regulated by viral protein E2, which is a transcription factor that binds to the early promoter p97 at specific E2-binding sites (E2BSs) located within the long control region (LCR) of HPV and can repress the expression of HPV *E6* and *E7* oncogenes (20,21), **Figure 3**. Overexpression of *E6* and *E7* can be caused either by disruption of *E2* gene via HPV genome integration or by inhibition of E2 protein binding to the LCR via HPV methylation (85). Therefore, viral integration and methylation are considered as two main regulatory mechanisms for malignant transformation (23,24,91,92), **Figure 7** and **Figure 8**.

Integration of HPV into the genome of an infected host cell has been extensively characterized in cervical cancers that are the widely acknowledged as HPV-associated cancers (96,97). In HNC, studies are mostly limited to cancer cell lines with few studies in patient specimens (83,100-105) and data on premalignant lesions are limited. While viral integration is thought to play an important role in cervical cancer (92), the relevance of viral integration is controversial in head and neck carcinogenesis (100). My first objective in this chapter is to address the role of viral integration in the development of cancers of the head and neck by determining the integration status in histologically distinct premalignant and malignant lesions.

Methylation of the HPV genome, which contains 15 CpG sites in the long control region (LCR), has been suggested as a biomarker for cervical cancer progression (23,24). Hypermethylation within HPV-LCR has been reported in cervical cancers or high- grade cervical dysplasia compared with low-grade CIN (23,24). In cervical cancer, it is shown that methylation of HPV inhibits binding of the transcriptional repressor (E2), resulting in overexpression of viral oncoproteins and leading to malignant transformation. However, very few studies have evaluated HPV methylation status in HNC specimens and their results are

inconsistent (111,112). To date, there is no information about the methylation pattern in premalignant hgOED lesions. My second objective in this chapter is to address the role of HPV DNA methylation in malignant transformation in head and neck tumors.

Toward these ends, I analyzed the physical integration of HPV DNA into the host chromosomes and presence of potential DNA methylation sites in the HPV epigenome in patients with premalignant and malignant tumors of the head and neck. Profiling of these genetic and epigenetic events may help to better understand the complex mechanisms of HPV-mediated head and neck carcinogenesis.

Experimental Procedures

Tissue specimens and cell lines: This study was approved by the IRB at the University of Louisville. Premalignant specimens from 40 patients were obtained from Biorepository of University of Louisville Oral Pathology Laboratory (as mentioned in Chapter II). HNC specimens from 50 patients were collected from Cancer Database and Specimen Repository at the James Graham Brown Cancer Center (as mentioned in Chapter II subheading 3). HNC cell lines were established at the University of Michigan and carry the heading, University of Michigan Squamous Cell Carcinoma (UMSCC). The HPV-negative cell line (UMSCC-1) and HPV-positive cell lines (UMSCC-47 and UMSCC-104) were purchased from EMD Millipore Corporation (Temecula, CA, USA), and cultured using standard protocols. Cervical cancer cell lines, CaSki and SiHa, were cultured as suggested by American Type Culture Collection (ATCC, Manassas, VA, USA).

DNA extraction and HPV detection: DNA was extracted from fresh frozen or FFPE specimens using DNAeasy Blood & Tissue kit (Qiagen, USA) as per manufacturer's instructions with RNAase (20 μl of 20mg/ml) treatment. FFPE samples were deparaffinized and washed before DNA extraction. HPV detection and genotyping were performed, as previously described (126). β-globin was used as an internal control. Sixteen of the 50 HNC specimens were HPV16 positive (**Table 7** for demographics). In premalignant lesions, HPV16 DNAs were present in 12/16 of Group 1, 7/14 of Group 2 and 2/10 of the Group 3. HPV type 16 is known to be the most common carcinogenic HPV type in cervical cancer development.

Similarly, HPV16 was the predominant form of HPV in specimens and cell lines derived from head and neck tumors. I used HPV16-positive premalignant and malignant samples for further analysis.

HPV integration and viral load by quantitative PCR: I employed a previously described real-time quantitative PCR assay to evaluate viral load and integration in cell lines and HNC specimens (165). The primers and probes were designed for specific amplification of the *E2* hinge regions which are known to be disrupted most frequently during the process of viral integration (165). *E6* primers and probes were also designed accordingly. β-globin was used as an internal control. In a total volume of 20 µl, the final primer, probe and DNA template concentrations were 0.3 µM, 0.1 µM, and 20 ng respectively. TaqMan™ Universal Master Mix II, with UNG (Uracil-N-glycosylase), was used according to the manufacturer's instructions (Thermo Fisher Scientific, USA). HPV16 plasmid cloned in a pBR322 vector (300 pg to 0.3 pg) was used to plot a standard curve. The relative viral load was estimated by calculating the ratio of a copy of *E6* found in the specimen to copy of *E6* present in SiHa cell because SiHa cells are known to contain a well-defined viral copy number (165). HPV integration status was evaluated using *E2*/*E6* ratio. The ratio of *E2* to *E6* equal or greater than 1 indicates the presence of HPV DNA in the episomal state with no integrated forms of viral DNA. An *E2*/*E6* ratio of 0 indicates HPV DNA in an integrated state with no episomal forms of viral DNA. An *E2*/*E6* of 0.5 shows the presence of equal copies of integrated and episomal forms of HPV DNA. Samples were categorized as predominantly integrated if the *E2*/*E6* ratio was less than 0.5 and predominantly episomal if the ratio was greater than 0.5. Cervical cancer cell lines (CaSki and SiHa) were used as positive controls for studying HPV genome integration in HNC cell lines and different head and neck tumor lesions. Water controls were included in each run. All experiments were performed in duplicates at least three times.

Determination of E2 Gene Integrity: The integrity of the *E2* gene was determined by amplification of the full-length *E2* ORF (nucleotide 2755 to 3852 of NC_001526.2; primers 16E2a and E2b listed in **Table 11**). Disruption of this region was defined as an absence of the full-length *E2* amplicon on agarose gel electrophoresis and positive signals of parallel β-actin amplification. Positive signals for amplification of the ~1 kb full-length *E2* amplicon from CaSki

DNA were used as a control because CaSki cells are known to carry an intact *E2* gene. Also, amplification of first half of *E2* (primers 16E2a and 16E2c) and last half of *E2* region (primers 16E2d and 16E2b) was performed for further confirmation of *E2* integrity.

Bisulfite-sequencing: Purified genomic DNA was bisulfite converted using an EpiTect ® Plus Bisulfite Kit (Qiagen, CA, USA) according to the manufacturer's protocol. In this treatment, unmethylated cytosine residues are converted to uracil whereas 5-methylcytosine is unaffected. Target DNA is then amplified by PCR in which uracil residues are converted to thymine. The DNA methylation status of HPV DNA was then evaluated by the direct sequencing of PCR products. Because HPV type 16 have different variants (categorized based single nucleotide polymorphisms present in *LCR* and/or *E6*), an Asian-American variant of HPV type 16 (Genbank accession number: AF402678.1) was used as reference HPV16 sequence for primer design and further analysis. The bisulfite-treated DNA was amplified by PCR using different sets of primers designed to target the 15 CpG sites in LCR region of HPV (**Figure 8**, **Table 11**). Primers were designed within the consensus sequences among different variants of HPV16 (Genbank accession numbers: AF402678.1, AF125673.1, AY686584.1, NC_001526.2, and KF954093.1). Different secondary primer sets were adopted in cases where PCR amplification was negative using primary primers. As an internal control for the presence of bisulfite-modified DNA, primers specific to a modified region of the β-actin (*ACTB*) gene containing no CpG sites was used. PCR reaction mixtures consisted of 10X HiFi PCR buffer, 50mM MgSO4, 10 mM dNTPs, 20 µM of each primer and 1 U of HiFi Taq polymerase (Thermo Fisher Scientific, USA) in a total volume of 20 µl. PCR conditions were 95°C (2 min); 45 cycles of 45 sec at 95°C, 45 sec at 52°C and 45 sec at 68°C, followed by 10 min at 68°C. PCR products were run on 3% agarose gel and extracted using QIAquick Gel Extraction Kit (Qiagen, USA) and sent for sequencing to the DNA Core Facility at the University of Louisville. Amplified products were directly sequenced using the same primers. Sequencing data was analyzed using NCBI BLAST database and SeqMan Pro program (Lasergene 12, DNAstar Inc., Wisconsin).

RNA extraction and qRT-PCR analysis: Total RNA was isolated from cultured UMSCC-1, 47 and 104 cells by using PureLink® RNA Mini Kit (Thermo Fisher Scientific, USA), with

DNase I treatment, according to manufacturer's instructions. Single-stranded complementary DNA (cDNA) was synthesized from 1 µg of total RNA using SuperScript® VILO cDNA Synthesis Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Quantitative real-time reverse transcription–polymerase chain reaction (qRT-PCR) was performed in separate 20-µL reaction volumes to evaluate the expression of HPV16 *E6*, *E7*, and *E2* genes, and cellular genes *p16 (CDKN2a/INK4a)*, *EGFR* and *β-actin*. To investigate the expression of *E2* gene, primers were designed near the 5' end and upstream of the frequent *E2* breakpoint to monitor the relative expression of truncated E2 mRNA. qRT-PCR was performed in triplicate using 100 ng of cDNA as template, the gene-specific forward and reverse primers (0.3 µM each) (**Table 11**), and the Power SYBR® Green Supermix (Thermo Fisher Scientific, USA) in an Applied Biosystems VIIa^{TM} 7 Real-Time PCR detection system (Thermo Fisher Scientific, USA). The amplification program for all primer sets was 95°C for 3 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Real-time PCR amplification data were analyzed and threshold cycle (Ct) numbers were automatically determined by VIIa^{TM} 7 software v1.2.4 (Thermo Fisher Scientific, USA). The relative expression of each mRNA was calculated by the ΔCt method (166,167). Endogenous *β-actin* mRNA levels were used for normalization of RNA expression. Due to the small amounts of RNA recovered from clinical biopsies, qRT-PCR was only performed on cell lines and not on clinical biopsy samples.

Statistical analysis: Comparisons were performed using Student's t-test or Fisher's exact test or Mann-Whitney Rank Sum Test. SigmaPlot Version 12.5 (Systat Software, Inc., San Jose California, USA) and Microsoft Excel 2013 (Microsoft Corporation, Redmond WA) were used for data computation and statistical analysis. Statistical significance was established at p-value ≤0.05 (*p<0.05; ** p<0.01; ***p<0.001).

Table 11: List of primers employed to detect HPV DNA, HPV integration, E2 gene integrity, bisulfite sequencing and qRT-PCR.

Target	Primer	Sequence (5'-3')
HPV detection		
L1	MY09* (For)	CGTCCMARRGGAWACTGATC
	MY11* (Rev)	GCMCAGGGWCATAAYAATGG
L1	GP5+ (For)	TTTGTTACTGTGGTAGATACTAC
	GP6+ (Rev)	GAA AAATAA ACTGTAAATCATATTC
β-globin	GH20 (For)	GAAGAGCCAAGGACAGGTAC
	PC04 (Rev)	CAACTTCATCCACGTTCACC
HPV integration by qPCR		
E2	16E2F	AACGAAGTATCCTCTCCTGAAATTATTAG
	16E2R	CCAAGGCGACGGCTTTG
	16E2probe	(FAM)-CACCCCGCCGCGACCCATA-(TAMRA)
E6	16E6F	GAGAACTGCAATGTTTCAGGACC
	16E6R	TGTATAGTTGTTTGCAGCTCTGTGC
	16E6probe	(FAM)-CAGGAGCGACCCAGAAAGTTACCACAGTT-(TAMRA)
E2 gene Integrity		
E2 (full length)	16E2a (For)	ATGGAGACTCTTTGCCAACGTT
	16E2b (Rev)	TCATATAGACATAAATCCAGTAGAC
E2 (first half)	16E2a (For)	ATGGAGACTCTTTGCCAACGTT
	16E2c (Rev)	TTATTCTTTGATACAGCCAGTGTTG
E2 (last half)	16E2d (For)	CCTCACTGCATTTAACAGCTCA
	16E2b (Rev)	TCATATAGACATAAATCCAGTAGAC
Bisulfite sequencing		
5'-LCR, enhancer	(2F+3R) For	GTGTATGTGTTTTTAAATGTTTGTGT
	(2F+3R) Rev	CACAATATACATAATAATTCAATAATTAC
enhancer	(4F+4R) For	GTAATTATTGAATTATTATGTATATTGTG
	(4F+4R) Rev	CACACACCCATATACAATTTTACAA
promoter	(5F+5R)For	TTGTAAAATTGTATATGGGTGTGTG
	(5F+5R) Rev	ACAACTCTATACATAACTATAATAACT
5'-LCR and	BSP-6 (For)	TAAATTATATTTGTTATATTTTGTTTTTGT
enhancer	BSP-6 (Rev)	TAATTAACCTTAAAAATTTAAACCTTATAC
β-actin	mACTB (For)	TGGTGATGGAGGAGGTTTAGTAAGT
	mACTB (Rev)	AACCAATAAAACCTACTCCTCCCTTAA
Gene expression by qRT-PCR		
E6	For	CAGCAATACAACAAACCG
	Rev	GCAACAAGACATACATCG
E7	For	CAGAGGAGGAGGATGAAATAG
	Rev	AGGTCTTCCAAAGTACGAATG
E2	For	TGATAGTACAGACCTACGTGACCATATAGA
	Rev	CCCATTTCTCTGGCCTTGTAAT
$p16^{INK4a}$	For	CATAGATGCCGCGGAAGGT
	Rev	CCCGAGGTTTCTCAGAGCCT
EGFR	For	GGAGAACTGCCAGAAACTGACC
	Rev	GCCTGCAGCACACTGGTTG
β-actin	For	CCATCGTCCACCGCAAAT
	Rev	GCTGTCACCTTCACCGTTCC
IPO8	For	CAGTGCATTCCACTCTTCGT
	Rev	ACGAAGCTCACTAGTTTTGACC

Results

Frequency of viral DNA integration in HNC cell lines

HPV16 DNA integration into the host genome is known to be strongly associated with HPV-induced cancer progression, and different integration patterns were reported in two cervical cancer cell lines (168). In this study, using an absolute quantification method with qPCR confirmed 2 copies of HPV16 DNA from SiHa cell line as previously reported (169). SiHa cells were then used as a reference for estimating the relative viral copy number in other cell lines and human tissue specimens (165). I found 325 copies of HPV16 DNA in CaSki, although different HPV DNA copy numbers (60–600) were previously reported in this cell line (169).

Integration of HPV16 DNA in the HNC cell lines and tumor tissues was examined using a method that was designed to provide a ratio of *E6* to *E2* gene copy number (165). The disruption of the E2 gene upon viral integration is known to be associated with HPV-induced cancer malignancy and was, therefore, the major focus of this study. The *E2*/*E6* ratio was 0.0 in SiHa, indicating that both copies of HPV16 DNA were integrated into chromosomal DNA on this cell line as reported previously (169). CaSki cells showed the *E2*/*E6* ratio of 0.12, indicating that the majority of HPV DNA in this cell line is integrated. These results were further confirmed by amplifying E2 ORF with nested PCR primers (**Table 11**). No full length of *E2* was found in SiHa while small 3'- or 5'- E2 fragments were amplified (**Figure 23C**). In CaSki, both full length and small fragments of E2 ORF were amplified confirming the presence of mixed forms of intact and disrupted E2 DNAs (**Figure 23**).

UMSCC-47 and 104 cell lines, originated from tumors of the anterior tongue and floor of the mouth, respectively, were classified as oral cavity cancers according to the anatomy-based classification of HNCs (170,171). HPV16 DNAs were detected from both cell lines while no DNA was found from the cell line UMSCC-1, which also originated from an oral cavity tumor. Relative to SiHa, the number of viral copies in HPV16-positive HNC cell lines (UMSCC-47 and UMSCC-104) was 2 (**Figure 26**). However, no full length of *E2* was amplified in both cell lines, suggesting both contained only integrated HPV16 DNA (**Figure 23**). HPV DNA integration was confirmed by evaluating *E2*/*E6* ratio from these cell lines.

E2/*E6* ratio values obtained from UMSCC-47 and UMSCC-104 (0.0 and 0.045 respectively) suggested that in both cell lines contained integrated HPV as the predominant species (**Figure 26**).

Characterization of HNC cell lines with integrated HPV DNA

Because the disruption of the *E2* gene upon HPV DNA integration causes robust and chronic expression of the viral *E6* and *E7* oncogenes in cervical cancer, the expression of *E2*, *E6*, and *E7* genes was profiled in HNC cell lines. Quantitative RT-PCR assays showed that E6/E7 mRNA levels varied considerably between the two HPV-positive HNC cell lines studied (**Figure 24**). *E7* expression was significantly higher in UMSCC-104 compared to UMSCC-47 ($p<0.001$); however the latter showed higher *E6* expression than the former ($p<0.05$). Expression of *E2* was analyzed using the primers designed to anneal upstream of the frequent *E2* breakpoint and both cell lines showed similar levels of *E2* expression. Additionally, the expression of $p16^{INK4a}$ and *EGFR* has been associated with HNCs (172,173), so their expression was also examined in HNC cell lines. The expression of $p16^{INK4a}$ was similar in HPV-positive HNC cell lines and significantly higher than that in the HPV-negative UMSCC-1 cell line. *EGFR* expression was relatively higher in UMSCC-104 than UMSCC-1, but was not significantly different between UMSCC-47 and UMSCC-1 (**Figure 24**).

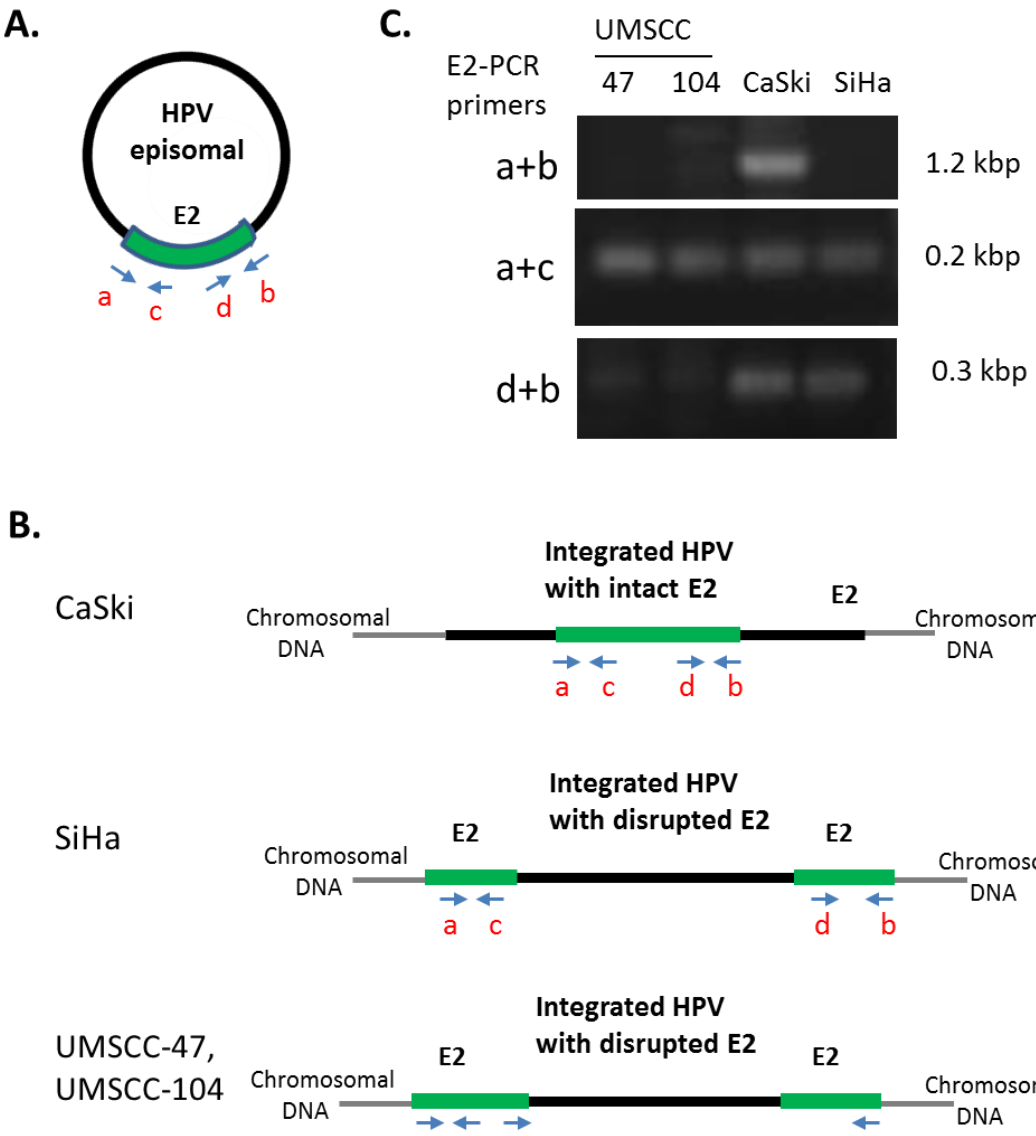

Figure 23: Determination of *E2* gene integrity **(A)** by using HPV16 E2 primers (16E2 a, b, c, and d, **Table 11**) which detect intact and disrupted *E2* gene. **(B)** Agarose gel image showing the full length of E2 in CaSki and disrupted E2 sequences in SiHa, UMSCC-47, and UMSCC-104. **(C)** Schematic representation of integrated HPV DNA in CaSki, SiHa, UMSCC-47 and UMSCC-104 cell lines.

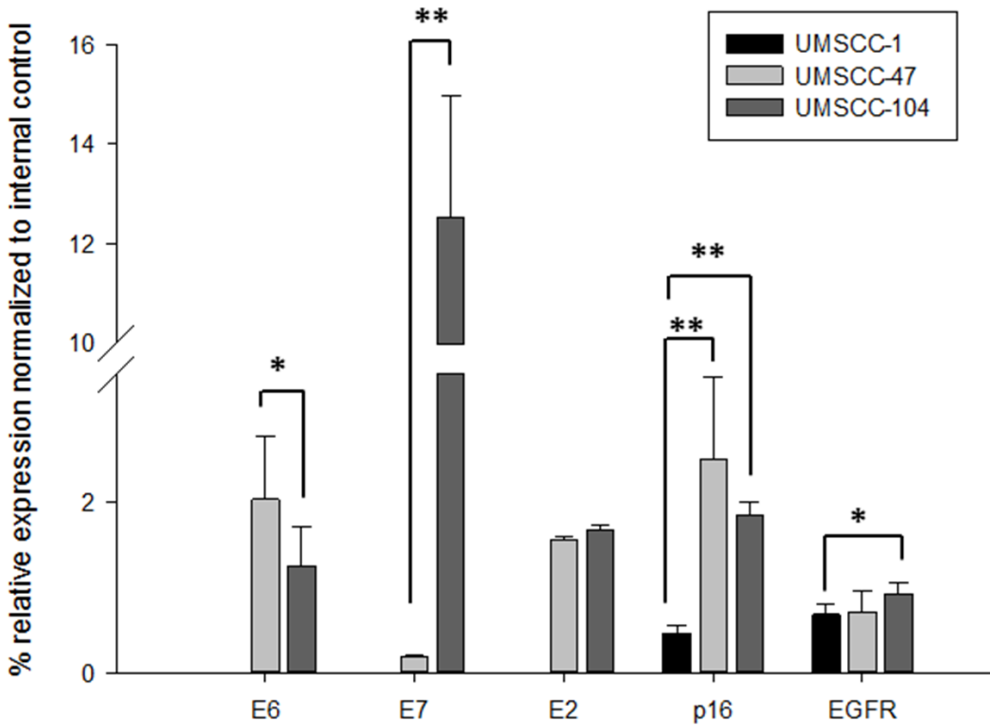

Figure 24: Relative expression of *E6*, *E7*, *E2*, *p16* and *EGFR* in UMSCC-47 and UMSCC-104 head and neck cell lines (Significance level at *p<0.05 and **p<0.01)

Differential HPV16 DNA integration rates in head and neck tumors collected at the different stages of malignant progression

In chapter II, I classified three different groups of premalignant oral samples based on their histological features. Briefly, Group 1 contained diffuse mitosoid/ apoptotic cells while in Group 2 and Group 3, histological features were focal or lacking. Group 1 was found to be strongly associated with the presence of HPV and higher levels of $p16^{INK4a}$ expression when compared with specimens in Group 2 and Group 3. Here, I evaluated HPV copy number and integration status to determine if there was a relationship between these parameters in the differently staged groups. HPV copy number was detected at variable levels in premalignant lesions and was significantly higher in Group 1 lesions compared to Group 2 ($p < 0.001$) and Group 3 ($p = 0.04$, Mann-Whitney test, **Figure 25**). Relative to SiHa, the HPV copy number in Group 1 lesions ranged from 1-47 with one exception of 0.1 copies. In contrast, all premalignant samples in Group 2 and Group 3 groups showed relative viral loads less than 0.1 (**Figure 25**). When I analyzed the integration state of HPV genome in HPV16 positive premalignant lesions, I observed a significant difference in the physical state of the viral DNA among the study groups ($p=0.006$, **Table 12**). The specimens in Group 1 contained a significantly higher level of integrated HPV DNA (90.9%) compared to Group 2 (28.6%) and Group 3 (0%). In contrast to Group 1, where integrated DNA was the predominant species, both episomal and integrated forms of HPV DNA were found in samples in Group 2.

HPV DNA copy number and integration status were also analyzed in 16 HNC specimens (malignant forms) and compared with those of premalignant tumors. Relative to SiHa, variable viral loads between 0.1 and 205 copies/cell (two cases had less than 0.1) were found in malignant specimens (**Figure 25**). 75% of these HNC specimens had integrated HPV as the predominant species, whereas only 6.25% of these HNC specimens had episomal DNA as the predominant species (**Table 12**). The patterns of HPV genome integration were similar between malignant and premalignant lesions of Group 1. Similarly, there was also no significant difference in viral load between Group 1 premalignant lesions and malignant specimens ($p= 0.79$).

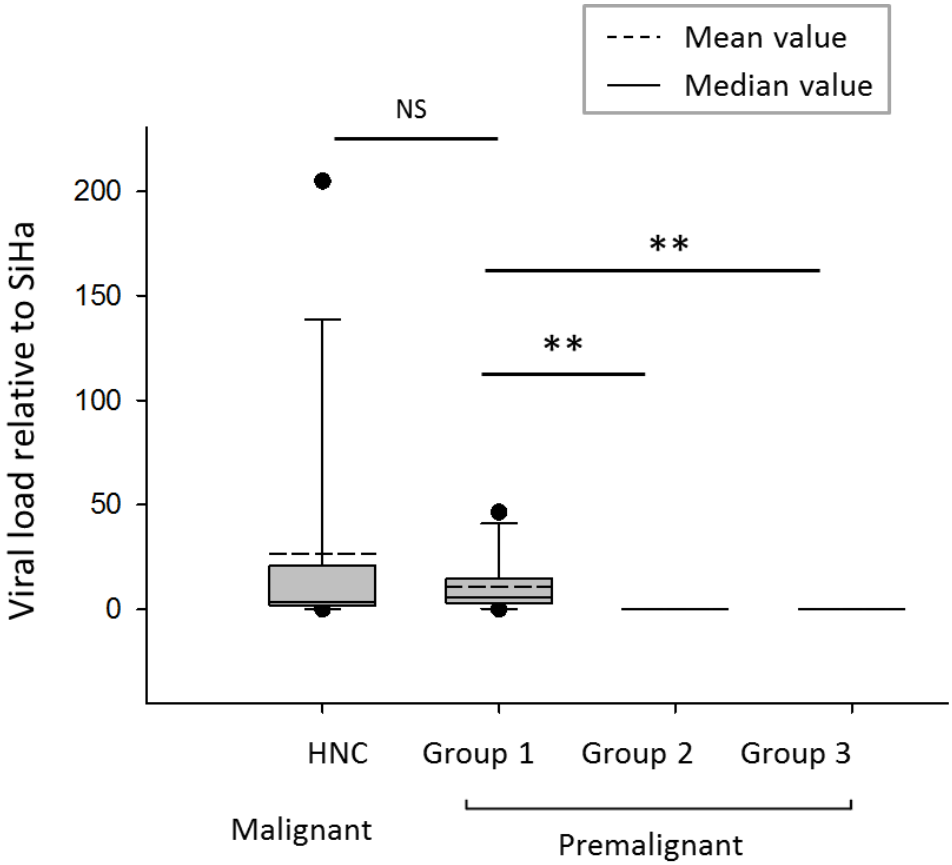

Figure 25: Box plot showing viral load in malignant (N=16) and different groups of premalignant [Group 1 (N=11), Group 2 (N=7) and Group 3 (N=2)] tumors, expressed in a number of times the level in SiHa cells. The bottom and top of the box show the first and third quartiles respectively, the solid line inside the box is the second quartile (*i.e.* the median), and the dashed line shows the mean value. Whiskers in the box plot represent the highest and lowest values excluding outliers as shown by dots. Long whisker in the case of the malignant specimen shows high variability in HPV copy number. In the case of Group 2 and Group 3 tumors, the values for mean, median and quartiles were almost same, so only solid lines are seen in the plot. (Significance level *p<0.05, **p<0.01, NS- non-significant, Mann-Whitney Rank Sum Test)

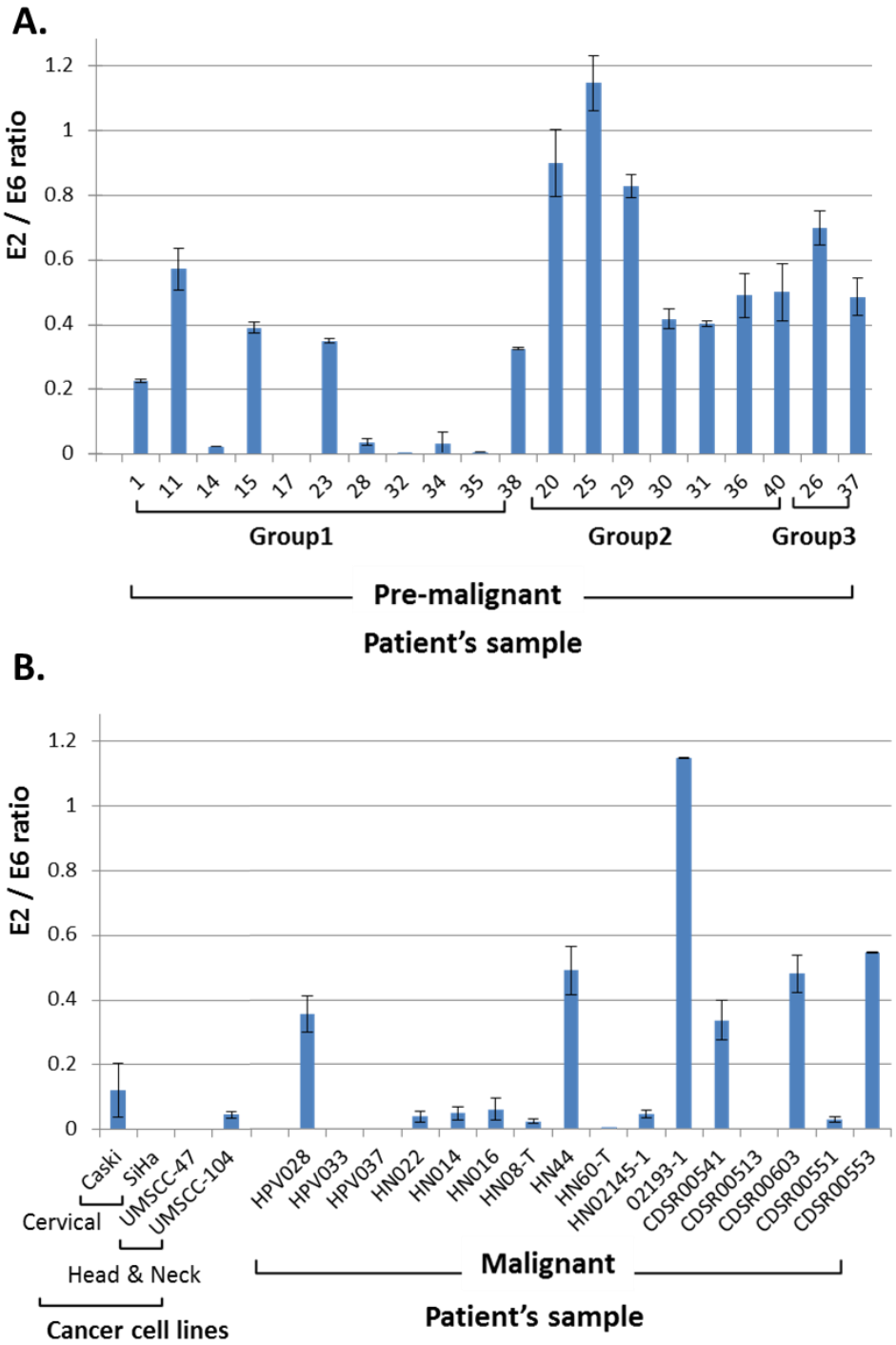

Figure 26: Assessment of HPV16 DNA integration by analyzing *E2*/*E6* ratio in **(A)** premalignant (Group 1, Group 2 and Group 3) tumors and **(B)** cancer cell lines (cervix-CaSki and SiHa; and head and neck, UMSCC-47 and 104), and malignant head and neck tumor specimens.

Table 12: HPV DNA integration in HPV16 positive premalignant hgOED and HNCs

HPV DNA integration status	Premalignant hgOED				HNC (malignant)
	Group 1	Group 2	Group 3	Overall p-value	
Solely or predominantly integrated	10 (90.9%)	2 (28.6%)	0 (0.0%)	**0.006**	12 (75%)
Episomal or predominantly episomal	1 (9.1%)	3 (42.9%)	1 (50%)		1 (6.25%)
Both episomal and integrated	0 (0.0%)	2 (28.6%)	1 (50%)		3 (18.75%)

Table 13: Methylation pattern of HPV16 cervical and HNC cell lines and HNC specimens*****

Sample* | CpG sites	**% Methylation**			**E2BS1**		**Enhancer**					**E2BS2**	**SP1**	**E2BS3**		**E2BS4**	
		7428	7434	7455	7461	7535	7553	7676	7682	7694	7862	31	37	43	52	58
SiHa	0	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U
CaSki	93.33	M	M	M	M	M	M	M	M	M	U	M	M	M	M	M
UMSCC-47†	76.92	M	mutated	M	M	M	M	U	U	M	U	mutated	M	M	M	M
UMSCC-104	0	U	U	U	U	U	U	U	U	U	U	U	U	U	U	U
HN08-T	93.33	M	M	M	M	M	M	M	M	M	U	M	M	M	M	M
HPV037	60.0	M	M	M	M	U	U	U	U	U	U	M	M	M	M	M
HN022	26.67	U	U	U	U	U	M	U	U	U	U	M	U	U	M	M

U denotes unmethylated; M denotes methylated.

***The remaining 13 HNC and 20 premalignant hgOED specimens were unmethylated at all 15 CpG sites.**

†UMSCC-47 only has 13 CpG sites due to two point mutations at nucleotides 7434 CG>CA and 31 CG>TG.

HPV LCR methylation in head and neck malignant progression

Methylation of the HPV genome has emerged as a biomarker for cancer progression in cervical cancer (23,24). To determine the role of HPV methylation in head and neck carcinogenesis, I first localized CpG sites within the HPV16 LCR. This region contains a promoter for transcription of viral early genes that includes E2 binding sites (E2BS) 1 to 4 and an SP-1 binding site (**Figure 8** and **Table 13**). There were 15, 15, 13 and 15 CpG sites in SiHa, CaSki, UMSCC-47 and 104 LCRs, respectively. UMSCC-47 had less CpG numbers than other cell lines tested because of its two point mutations [nucleotide (nt.) 7434 CG>CA and nt. 31 CG>TG], as reported previously (174). A variety of methylation patterns on the promoter within the LCR were found in four HPV cancer cell line derived from HPV16 infections. CaSki and UMSCC-47 cells showed hypermethylation (93.33% and 76.92% of available CpGs, respectively). The CpG at nt.7862 within E2BS-2 was unmethylated in both cell lines. UMSCC-47 had additional unmethylated CpGs at nucleotides 7676 and 7682. In contrast, UMSCC-104 and SiHa cell lines showed unmethylated CpG at all 15 sites within LCR. Consequently, two different HPV cancer cell lines (CaSki and UMSCC-47) showed hypermethylation and the other two (SiHa and UMSCC-104) showed hypomethylation patterns.

The methylation patterns on viral LCR were then profiled in premalignant and malignant lesions of the head and neck in specimens where DNA integration rates were analyzed (**Table 13**). The methylation patterns of CpG were not different among premalignant specimens between the histologically distinct groups. Indeed, all CpG sites within the LCR were unmethylated in all premalignant specimens. Moreover, all HNC specimens were predominantly unmethylated, except three malignant samples which contained methylated CpG (93.33%, 60%, and 26.67% methylation, respectively). From these results, I observed overall hypomethylation pattern of HPV epigenome from samples of HNC patients.

Discussion

The molecular determinants of HPV-associated HNC development and progression are not well-defined. Viral integration and methylation could potentially play a role in HPV-induced head and neck carcinogenesis as seen in HPV-associated cervical cancer. However,

limited studies have addressed this highly relevant issue and are mostly done using HNC cell lines with few studies in patient tumor samples and even fewer in pre-malignant specimens. In this study, I have analyzed histologically characterized HPV-positive tumors, collected at different stages of malignancy, with a goal of elucidating mechanisms of HPV-associated head and neck malignancy.

I used cervical and head and neck cancer cell lines to collect reference data that are useful for the analysis of clinical samples. HNC cell line UMSCC-47 contained integrated HPV with disruption of the E2 gene, similar to SiHa. Further, UMSCC-104 showed a low *E2*/*E6* ratio with no amplified product for the full length of *E2* (**Figure 23**), indicating that HPV had integrated (**Figure 26**). These results were consistent with data from other researchers (168) . However, one study suggested the presence of only episomal HPV in UMSCC-104 (175). This discrepancy might be due to the use of a different set of primers that do not target full-length *E2*. According to Akagi *et. al*, in UMSCC-104, the integration breakpoint is at the far 3' end of *E2* gene, which differs from the frequent breakpoint site as seen in SiHa and UMSCC-47 (168). This unexpected location of the breakpoint likely explains why this was missed by Olthof *et. al* (175).

Methylation is another mechanism by which expression of the HPV oncogenes can be reduced and contribute to malignant progression (176-179) , therefore I also studied promoter methylation in HPV cell lines and patient samples. I began by studying promoter methylation in HPV-positive HNC cell lines (UMSCC-47 and -104) and compared with that in cervical cancer cell lines (CaSki and SiHa). The SiHa cell line where HPV DNA had integrated was hypomethylated within the LCR, whereas CaSki containing both integrated and episomal HPVs was hypermethylated (**Table 13**, **Figure 23** and **Figure 26**). Surprisingly, the pattern of methylation and its correlation with integration status showed exactly the opposite in the two HNC cell lines: UMSCC-47 with only integrated DNA (similar to SiHa) showed hypermethylation pattern (resembling CaSki), and UMSCC-104 containing predominantly integrated HPV DNA showed hypomethylation pattern (**Table 13**, **Figure 23** and **Figure 26**). Therefore there was no correlation between integration status and promoter methylation of HPV DNA between the different cancer cell lines used in this study.

As mentioned earlier, viral integration and methylation are considered as two different mechanisms to interfere with E2 function thereby increasing oncogene expression and potentially leading to malignant transformation. Both HPV- positive HNC cell lines used in this study showed integrated HPV DNA, therefore it was not possible to correlate viral oncogene expression with integration status. However, the UMSCC-104 cell line which was derived from a recurrent tumor showed significantly higher *E7* expression compared to UMSCC-47. In contrast, the UMSCC-47 cell line with hypermethylated HPV showed higher *E6* levels than UMSCC-104, which contained hypomethylated HPV DNA. These data show no clear pattern relating promoter methylation to oncogene expression, except to possibly suggest that there may be differences between oncogenes in this regard. More studies are necessary to determine whether changes in methylation patterns in the LCR influence the expression of the HPV oncogenes, *E6* and *E7*.

Overexpression of $p16^{INK4a}$ has been correlated with HPV-positive cancers (172,173). As expected, $p16^{INK4a}$ expression was significantly higher in both HPV- positive HNC cell lines compared to HPV-negative UMSCC-1 cell line. *EGFR* is also frequently overexpressed in HNC independent of HPV etiology (180,181). Overexpression of *EGFR* is considered a poor prognostic factor in HNCs (182). Accordingly, compared to UMSCC-1 (which is HPV-negative HNC cell line), I found significantly higher *EGFR* expression in UMSCC-104 cells which was isolated from an HPV-positive tumor that did not respond to treatment (170).

Integration of high-risk HPV DNA into host cells in severe dysplasia or carcinoma *in situ* of the cervix has been suggested as the first epidemiological marker of malignancy (183-186). Therefore, for the first time, I analyzed HPV integration status in HPV16-positive samples collected at different malignant stages of tumors from the head and neck region, mainly by incorporating pre-malignant lesions in the study. I found that HPV integration status differed significantly between histologically distinct premalignant hgOED groups. Although the consequences of HPV DNA integration in oral dysplastic cells remains unexplored, I found that Group 1 (having diffuse mitosoid/ apoptotic cells) was more likely to have integrated HPV DNA (90.9%) than Group 2 and Group 3 (where histological features were focal or lacking). Group 1 had mostly integrated HPV DNA, which may confer higher malignant transformation

potential of Group 1 to HPV-associated cancer than Group 2 or Group 3. Therefore, the presence of an abundance of mitosoid cells in any premalignant squamous lesion could indicate the potential development of an HPV-associated oral cancer, though further research is warranted to confirm this hypothesis. In the malignant specimens, I also observed integration of HPV DNA in a significant proportion of HPV-positive HNCs, which was as expected based on the results from cell lines. These observations obtained from malignant specimens were also consistent with the results from other studies (104,105).

In the case of cervical cancerous and pre-cancerous lesions, higher CpG methylation in E2BS was seen in samples containing only episomal HPV16 compared to those with integrated HPV16 genomes (178). So, HPV LCR methylation was considered as an alternative mechanism to HPV integration for cervical malignancy. However, HPV epigenome of head and neck tumors was mostly hypomethylated in contrast to what was observed in cervical lesions (23,24). This is also in contrast with a small study consisting of 3 HPV-positive HNCs, which showed hypermethylation in LCR regions (111). However, a study in a large number of malignant samples reported hypomethylation of LCR in OPSCC (112), which is consistent with the results obtained in this study. Moreover, my analyses showed no significant differences in the methylation pattern between head and neck premalignant and malignant specimens with or without integration. In malignant specimens containing predominantly episomal HPV DNA, I didn't find any methylation for CpG sites. Additionally, since all 15 CpG in premalignant specimens were unmethylated, I could not find any difference in methylation pattern among 3 groups, although they varied in integration status. Consequently, my results indicated that integration status of tumor lesions of the head and neck did not seem to correlate with HPV methylation pattern. This warrants further extensive research to determine the relationship of HPV genome methylation and integration in head and neck carcinogenesis.

Overall, this is the first study to show differential HPV integration pattern in histologically distinct premalignant samples. This study revealed that HPV DNA integration might play a role in the process of HPV-induced head and neck carcinogenesis but not HPV methylation.

CHAPTER V

HOST EPIGENETIC CHANGES BY HPV INFECTION

Chapter Overview

DNA methylation is an important epigenetic modification in human cells. Both viral genomic and host chromosomal DNA can undergo methylation. In chapter IV, I found that promoter methylation of HPV DNA (HPV epigenome) gave no consistent patterns in head and neck cancer. In this chapter, I focused on characterization of host epigenetic changes to better understand cancer progression driven by HPV infection in the head and neck. For this study, an array of tumor suppressor genes was selected based on the data available from PubMed that seem to be implicated in HPV carcinogenesis. Results of my study showed that *EREG* is a candidate target gene for epigenetic regulation by HPV in HNC cells.

Introduction

Epigenetic modification, such as DNA methylation, histone modification, chromatin remodeling and microRNA, are essential for normal cellular differentiation, development and gene expression (187). Therefore, abnormal epigenetic modifications may contribute various pathological states. In particular, gene silencing through aberrant promoter methylation is considered as an important cause of tumorigenesis (118,188). Unmethylated cytosines in CpG island within the promoter region allows gene expression while methylated CpG causes silencing of genes. Methylated CpG binds with methyl-CpG-binding proteins and in turn, recruit histone deacetylases, resulting in transcriptional inactivation and loss of gene expression, as described in **Figure 9** (113,189). Methylation of DNA is catalyzed by a group of enzymes called DNA methyltransferases (DNMTs), **Figure 9A**. DNMT1 is mainly responsible for maintenance methylation in which the methylation pattern is transmitted during cell division (190). The DNMT3 group of methyltransferases, particularly DNMT 3a and

DNMT3b performs *de novo* methylation in which previously unmethylated cytosines are modified (113). There is growing evidence that the DNA methylation is a target for epigenetic changes induced by HPV *E7* and *E6* oncogenes (119,191). It has been shown that HPV16 infection is associated with an up-regulation of DNMT1 and DNMT3a (119). Therefore, different studies related to epigenetic alterations have been largely investigated in cervical cancers, where hypermethylation of viral and host genes were frequently detected in advanced cancers (86-90,192). Accordingly, epigenetic alteration (particularly, promoter hypermethylation of the tumor suppressor genes) are known to play a major role in the development of HNCs (121,122,125,193,194). Although studies have shown epigenetic modulation in HNCs (121,122,195), it is unknown the extent to which these changes contribute to HPV-mediated carcinogenesis and progression. A better understanding of the epigenetic differences between HPV-positive and HPV-negative HNCs will help to identify molecular signatures involved in HPV-mediated HNC carcinogenesis.

In order analyze the methylation status of tumor suppressor genes (TSGs) and identify epigenetic changes specific for HPV-positive HNCs, I surveyed publicly available resources- NCBI Epigenomics and PubMed literature. Thereby, I selected 38 candidate TSGs thought to be hypermethylated and repressed on cervical cancers and HNC (125,193-210). There were only a few reports on the relationship between HPV infection and methylation of host genes in HNCs (104,195,211-215) and results varied among studies. Herein, I hypothesized that presence of HPV infection leads to methylation of host genes and thereby reduces expression of distinct unique tumor suppressor genes in HNCs.

Experimental Procedures

Cell culture: Head and neck cancer cell lines; UMSCC-1 (HPV-negative) and UMSCC-47 (HPV-positive); were purchased from EMD Millipore Corporation (Temecula, CA, USA) and cultured as per manufacturer's instructions. UMSCC-1 and -47 originated from the floor of the mouth and anterior tongue, respectively, such that they are classified as oral cavity cancers (subsets of HNCs).

DNA isolation: DNA was isolated from cultured UMSCC-1 and 47 cells using DNAeasy® Blood & Tissue kit (Qiagen, USA) as per manufacturer's guidelines. The same method was applied to isolate DNA from 5-aza-2′-deoxycytidine treated cells.

RNA isolation and cDNA synthesis: Total RNA was extracted from all samples by using PureLink® RNA Mini Kit (Thermo Fisher Scientific Inc, USA), with DNase I treatment, according to manufacturer's instructions. To detect mRNA expression, single-stranded complementary DNA (cDNA) was synthesized from 1 µg total RNA by using SuperScript® VILO cDNA Synthesis Kit (Thermo Fisher Scientific Inc, USA) according to the manufacturer's guidelines.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Analysis: Quantitative RT-PCR was conducted on a ViiA7 Real-Time PCR system (Applied Biosystems, Thermo Fisher Scientific Inc, USA). Expressions of DNMTs were quantified using Power SYBR Green PCR Master Mix (Thermo Fisher Scientific Inc, USA) and normalized against expression of a β-actin housekeeping gene. Each sample was measured two times at least and fold changes in mRNA expression levels were calculated using the comparative threshold (Ct) cycle method. Expressions of DNMTs were also measured using pre-designed TaqMan primers and probes in TaqMan array plate. Results were consistent from both Sybergreen assay and TaqMan array assay plate.

Quantification of global DNA methylation: DNA was isolated as described previously in earlier chapter II. Global methylation levels were assayed using Methylamp™ Global DNA Methylation Quantification Ultra Kit (Epigentek) following the manufacture's protocol. In this assay, DNA is immobilized to the well which is specifically coated with DNA binding substance. The methylated fraction of DNA is recognized by 5-methylcytosine antibody and quantified through an ELISA-like reaction.

Selection of endogenous control for Taqman gene expression studies: TaqMan® Human Endogenous Control array (Applied Biosystems, Thermo Fisher Scientific Inc, USA) was used to identify appropriate endogenous controls that are invariably expressed in different samples being compared (*i.e.* 5-aza-dc treated and untreated UMSCC-1 and UMSCC-47 cells). This array card contains a panel of 16 endogenous control gene

candidates (probe/primer sets preloaded into the array card), **Table 14**. cDNA from each sample were synthesized from 1 µg total RNA and equal concentration (200 ng in each well) were loaded in triplicates into the endogenous control array card according to manufacturer's specifications, centrifuged and run on a ViiA7 Real-Time PCR system (Applied Biosystems, Thermo Fisher Scientific Inc, USA). Accordingly, three genes- namely 18S rRNA, GAPDH, and IPO8 were selected (since their standard deviations were less than 0.5 Ct) and added in TaqMan custom array plate.

Customized TaqMan® Array Plate: Assorted from various previous studies, 38 tumor suppressor gene (TSG) candidates that showed to be hypermethylated with decreased expression in cervical and HNCs were selected and added in custom array plate (**Table 15**). In addition, the plate also contains three DNMTs (DNMT1, DNMT3a, and DNMT3b); four HPV-affected genes (TP53, RB1, $p16^{INK4a}$, EGFR) and three internal control genes (18S, GAPDH, IPO8; which was selected from TaqMan Endogenous Control Arrays). Relative changes in gene expression in UMSCC-47 versus UMSCC-1 and relative changes on 5-aza-dc treatment as compared to untreated (DMSO treated) control cells was analyzed using normalization with IPO8.

5-aza-2'-deoxycytidine (5-aza-dc) treatment: The de-methylation reagent 5-aza-dc treatment assay was used to analyze the functional effect of gene methylation on its expression in HNCs. 5-aza-dc (Sigma Aldrich Inc., St. Louis, MO) was dissolved in DMSO to make a stock solution of 20 mM and stored in aliquots at −80°C until use. From the stock solution, it was diluted directly in the culture medium to obtain desired final concentrations. Cells were treated with 5-aza-dc at different concentrations (0.5–30 µM) for 96 h. The culture medium containing freshly prepared 5-aza-dc was replaced every 24 h. Control cells were treated in parallel with DMSO only. Cell viability and global methylation were tested to determine optimal 5-aza-dc concentration for each cell type. Cells were harvested, and DNA and RNA were isolated.

Cell viability: Cell viability before and after 5-aza-dc treatment was assessed by crystal violet staining method. Briefly, cells were seeded in each well of a 24-well plate and were allowed to grow for 24 h or 70 % confluence before treatment. Cells grown in monolayer were treated

with various doses of 5-aza-dc for 96 h. After treatment, cells were washed with PBS, fixed with ice-cold methanol and stained with 0.5% crystal violet (prepared in 50% methanol) for 15 mins and then washed with PBS and air-dried for 10 mins. Cells were then destained with 10% acetic acid for 5 mins in the rocker. Absorbance was measured at 595nm using Gen5 Microplate Reader and Imager Software (BioTek Instruments, Inc., VT, USA). For the evaluation of cell viability, the average absorbance of the control (5-aza-dc untreated) wells was regarded as 100%, and the percentage of cell growth in each well was calculated (% of control).

Promoter Methylation Detection: DNA samples were subjected to bisulfite treatment using an EpiTect ® Plus Bisulfite Kit (Qiagen, CA, USA) following the manufacturer's protocol. In this treatment, methylated cytosine remains unaffected whereas unmethylated cytosine residues are converted to uracil, which gets converted to thymine during PCR amplification. Methylation-specific PCR (MS-PCR) was performed to identify promoter methylation of *EREG*, *GRB7*, *SMG1*, *RUNX3*, and *CHFR* in HNC cell lines. Ensemble database was used to find and retrieve promoter sequences of these genes. Nucleotide sequences from ~1 kbp 5'-flanking sequence to ~500 bp downstream of the transcription initiation site (TSS) of the gene was entered into MethPrimer program (http://www.urogene.org/methprimer/) (216). Primers specific for methylated sequences and unmethylated sequences of the gene were designed using MethPrimer program (**Table 16**). To control for the presence of bisulfite-modified DNA, primers specific to a modified region of the β-actin (ACTB) gene containing no CpG sites were used (mACTB Forward primer: TGGTGATGGAGGAGGTTTAGTAAGT and Reverse primer: AACCAATAAAACCTACTCCTCCCTTAA). The amplified products were resolved on 3% agarose gel and visualized under Bio-Rad ChemiDoc™ imaging system using ChemiDoc™ XRS software. Results of promoter methylation of *SMG1, RUNX3* and *CHFR* are included in an appendix so as not to distract from the focus of the study.

Statistical analysis: Data was analyzed by Students t-test or Analysis of Variance (ANOVA). One-way ANOVA or Student's t-test was employed to test the effect of treatment on expression of each gene on each cell line individually. Two-way ANOVA was done to test the effect of treatment across the two cell types for each gene (*i.e.* the interaction between

"Treatment" and "Cell type"). Bonferroni p-value correction was designed to test the interaction effect across seven genes and to correct the Type 1 error rate (*i.e.* false positive result) for multiple comparisons. The analysis was performed using SigmaPlot Version 12.5 (Systat Software, Inc., San Jose, CA, USA), GraphPad Prism Version 7 (GraphPad Software, Inc., La Jolla, CA, USA) and Microsoft Excel 2013 (MS Corp, Redmond WA). P values ≤ 0.05 were considered statistically significant (*p<0.05, **p<0.01 and ** p<0.001). Error bars represented standard deviations of the mean fold change.

Table 14: Endogenous control genes contained on Applied Biosystems (ABI) Human Endogenous Control Array

Gene name	**Gene symbol with ABI product reference number**
Eukaryotic 18S rRNA	18S-Hs99999901_s1
Actin, beta	ACTB-Hs99999903_m1
Beta-2-microglobulin	B2M-Hs99999907_m1
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH-Hs99999905_m1
Glucuronidase, beta	GUSB-Hs99999908_m1
Hydroxymethylbilane synthase	HMBS-Hs00609297_m1
Hypoxanthine phosphoribosyltransferase 1	HPRT1-Hs99999909_m1
Importin 8	IPO8-Hs00183533_m1
Phosphoglycerate kinase 1	PGK1-Hs99999906_m1
Polymerase (RNA) II polypeptide A, 220kDa	POLR2A-Hs00172187_m1
Peptidylprolyl isomerase A (cyclophilin A)	PPIA-Hs99999904_m1
Ribosomal protein, large, P0	RPLP0-Hs99999902_m1
TATA box binding protein	TBP-Hs99999910_m1
Transferrin receptor (p90, CD71)	TFRC-Hs99999911_m1
Ubiquitin C	UBC-Hs00824723_m1
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	YWHAZ-Hs00237047_m1

Table 15: Genes present in the custom TaqMan Array Plate including three endogenous controls (18S, GAPDH and IPO8)

18S	GAPDH	IPO8	DNMT1	DNMT3A	DNMT3B
TP53	RB1	EGFR	CCNA1	GRB7	CDH11
CDH8	CDH13	PCDH10	PAX1	SFRP4	SMG1
MT1G	PHF21B	RUNX3	RUNX2	SPON2	PREX1
IRS1	MGMT	RASSF1	CADM1	TIMP3	CHFR
DAPK1	S100A2	RARB	APC	DCC	NDRG2
TUSC3	TCF21	GATA4	IRX4	ESR1	CDKN2A
CDH1	EDNRB	RASSF5	GALC	EREG	FHIT

Table 16: Methylation-specific PCR primers

Genes	**Primers for methylated sequence**	**Primers for unmethylated sequence**	**Amplicon size (bp);** (Methylated, M; Unmethylated, U)
EREG	Met-Forward GTTTTTTAGTTATTGTCGCGAGTTC Met-Reverse TTATCTCCTCTTTAAAACGACCG	UnMet-Forward TTTTTAGTTATTGTTGTGAGTTTGT UnMet-Reverse TTATCTCCTCTTTAAAACAACCAAA	154 (M) and 153 (U)
GRB7	Met-Forward TTTTTTATTATTTGTAGAGAAGCGG Met-Reverse AACACAACCTAAAACCCTAATACGA	UnMet-Forward TTTTTTATTATTTGTAGAGAAGTGG UnMet-Reverse ACACAACCTAAAACCCTAATACAAC	197 (M) and 196 (U)
CSF3	Met-Forward GATCGTGATTATTTTGGTTAATACG Met-Reverse CTCACTACAAACTCTACCTCTCGAA	UnMet-Forward ATTGTGATTATTTTGGTTAATATGG UnMet-Reverse CTCACTACAAACTCTACCTCTCAAA	154 (M) and 153 (U)
SMG1	Met-Forward GCGTACGTGAATTTAAGGGTAC Met-Reverse AACAAAAAATCTCCACTACTACGAC	UnMet-Forward GGTGTATGTGAATTTAAGGGTATGT UnMet-Reverse AACAAAAAATCTCCACTACTACAAC	177 (M) and 178 (U)
RUNX3	Met-Forward ATTTTTTGAAGGGTTGAAAATTTTC Met-Reverse CAAAACGATCAATAAAAAAACGTA	UnMet-Forward TTTTGAAGGGTTGAAAATTTTTG UnMet-Reverse CCAAAACAATCAATAAAAAAACATA	180 (M) and 178 (U)
CHFR	Met-Forward TTTTGTGATAATATATTTTTTTCGT Met-Reverse ATAATCTTAATCTCCTAACCTCGTA	UnMet-Forward TTTTTGTGATAATATATTTTTTTTGT UnMet-Reverse ATAATCTTAATCTCCTAACCTCATA	165 (M) and 166 (U)

Results

Increased DNMT1a and DNMT3a expression in UMSCC-47 cells

As an initial characterization of epigenetic modifications associated with HPV-positive HNC cells, DNA methyltransferase activity, and the global DNA methylation were quantified in UMSCC-47 (HPV-positive) and UMSCC-1 (HPV-negative) cells. Significantly higher expressions of *DNMT1a* and *DNMT3a* were found in HPV-positive UMSCC-47 cells as compared to HPV-negative UMSCC-1 cells (**Figure 27A**). This result supports the finding that shows up-regulation of DNMT1 and DNMT3a induced by HPV16 infection (119). However, *DNMT3b* expression was decreased in the UMSCC-47 cell line. As expected, global DNA methylation in UMSCC-47 cells was significantly higher compared to UMSCC-1 cells (**Figure 27B**). These results were consistent with a study done by Lechner *et al*. which showed increased mRNA expression of both DNMT1 and DNMT3a in HPV-positive HNC cell lines (213). Consistent results were also found in *E6*- and *E7*-transfected HNC cell lines (213).

Gene expression profiling of tumor suppressor genes in HPV-positive and negative HNC cells

In this study, I selected 38 tumor suppressor genes, which were reported previously to be altered in either cervical cancer or HNC; although it should be noted that HPV-specific alteration in the expression of host genes has been poorly characterized and results varied from one study to another. Some of these genes that were previously shown to be hypermethylated and/or inactivated in HNCs are -- *GRB7*, *CDH11*, *SFRP4* (212); *CDH8*, *CDH13*, *PCDH10* (213); *PAX1* (125); *SFRP4* (197); *SMG-1* (214); *MT1G* (198); *PHF21B* (199); *RUNX3* (200); *MGMT*, *RASSF1A* (194); *RASSF1* (201,202); *CADM1/IGSF4* (203); *DAPK1* (202,215); *S100A2* (206); *CDH1*, *EDNRB, RARB*, *APC*, *DCC*, *NDRG2* (193); *TUSC3* (207,212); *TCF21* (211); *GALC* (209); *EREG* (195); *FITH* (195,210). There were genes that were previously found to be hypermethylated and inactivated specifically in HPV-positive HNCs (or were more hypermethylated in HPV-positive HNCs than HPV-negative HNCs); such as *CCNA1* (211,212) *RUNX2*, *SPON2*, *PREX1*, *IRS1* (195), *TIMP3*, *CHFR* (215); *GATA4*, *IRX4* (104,208).

The expression levels of these 38 tumor suppressors were analyzed in a custom array plate that I designed. No significant changes in the expression of *CCNA1*, *SPON2*, *PREX1*, *IRS1*, *TIMP3*, *GATA* and *IRX4* were found between the HPV-positive (UMSCC-47) and HPV-negative (UMSCC-1) cell lines. Nevertheless, six genes (*GRB7*, *RUNX3*, *RUNX2*, *CHFR*, *RARB,* and *EREG*) showed significantly reduced expression in the HPV-positive HNC cell line compared with the HPV-negative HNC cell line (**Figure 28**). In addition, one gene, *SMG-1* was marginally reduced in HPV-positive cell line as compared to HPV-negative cell lines (p=0.08).

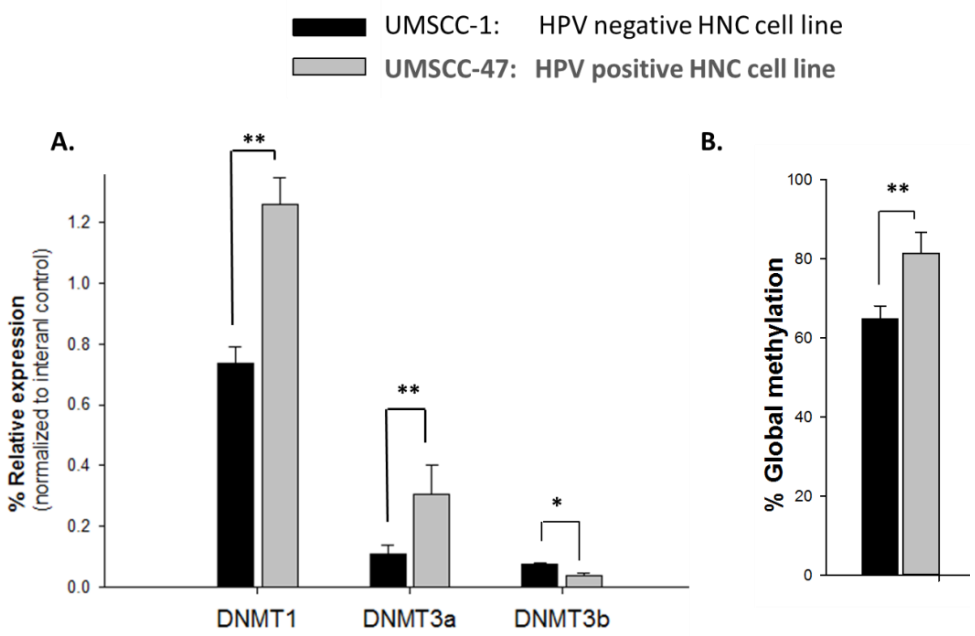

Figure 27: Relative expression of DNMT **(A)** and global methylation **(B)** in head and neck cancer cell lines. HPV-positive cell line (UMSCC-47) showed significantly higher DNMT1 and DNMT3a expressions and greater percentage global methylation than HPV-negative cell line (UMSCC-1). (Significance level at *p<0.05, **p<0.01 and NS non-significant)

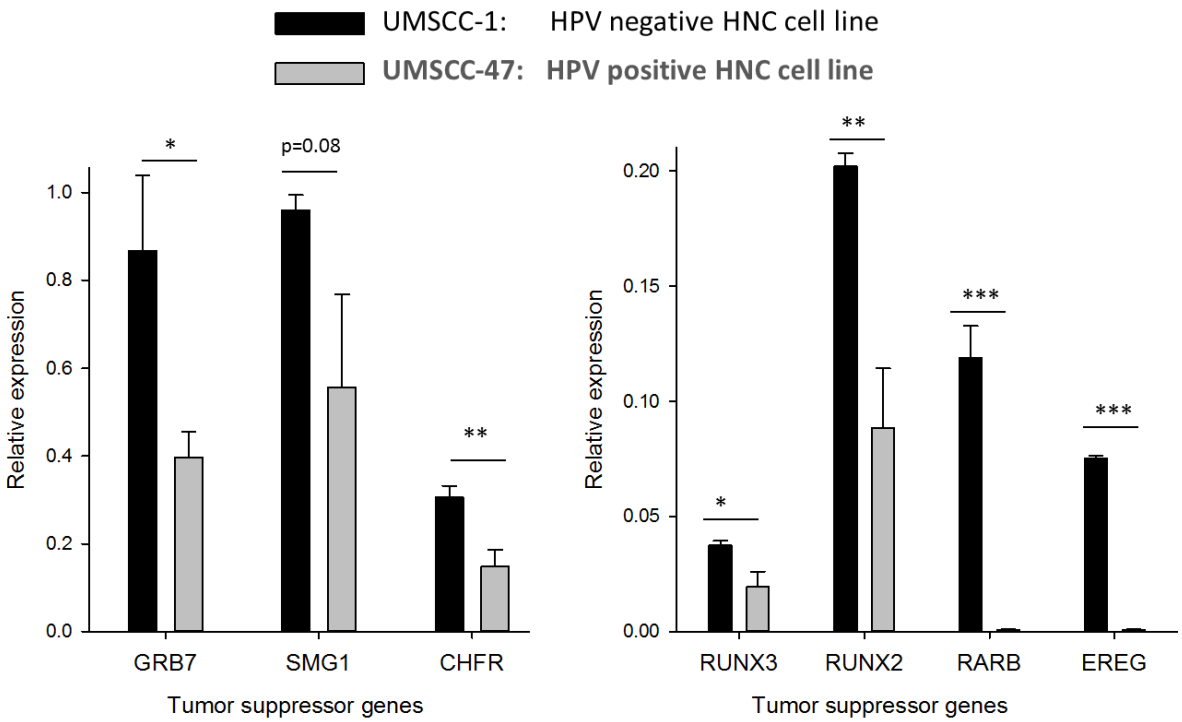

Figure 28: Gene expression profile of six tumor suppressor genes that showed reduced expression in HPV-positive cell line (UMSCC-47) as compared to that of HPV-negative cell line (UMSCC-1). (Significance level at *p<0.05, **p<0.01, **p<0.001 and NS non-significant)

Determination of optimal de-methylation condition using 5-aza-dc treatment

Seven tumor suppressor genes showing decreased expression in HPV-positive HNC cell line were selected to test whether their decreased expression corresponds with promoter methylation using the de-methylation agent (5-aza-dc). Initially, I determined optimal conditions for de-methylation using 5-aza-dc by testing cell viability and global methylation. When cells are treated with 5-aza-dc (a nucleoside analog of cytidine), it gets incorporated into DNA during replication forming azacytosine-guanine dinucleotide. DNA methyltransferase (DNMT) recognizes this dinucleotide as a natural substrate for methylation and subsequently gets covalently bound (*i.e.* trapped) to DNA, thereby inactivating its enzymatic function (217). Since 5-aza-dc does not actively de-methylate the DNA, but rather inhibits the methylation of new DNA during cell division, cells were treated with 5-aza-dc for a relatively long period of time (4 days with re-treatment every day due to poor stability of the drug). Further, since different cell lines vary in terms of toxicity to this drug, as well as their response in de-methylation, I treated cell lines with different concentrations (0.5–30 μM) of 5-aza-dc. I found

that treatment with 5 μM 5-aza-dc for 96 hours showed optimal de-methylation potency based on global methylation (67% and 75% reduction in global methylation in UMSCC-1 and 47 respectively) with ~60 and 70% cell viability (**Figure 29 and Figure 30**).

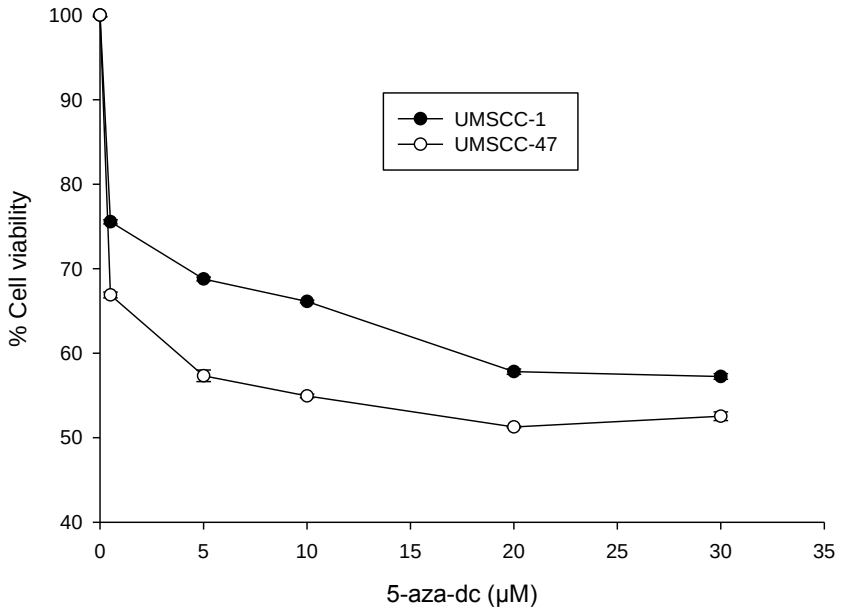

Figure 29: Cell viability of HPV-positive (UMSCC-47) and HPV-negative (UMSCC-1) cell lines with increasing dosage of 5-aza-dc treatment for 96 hours.

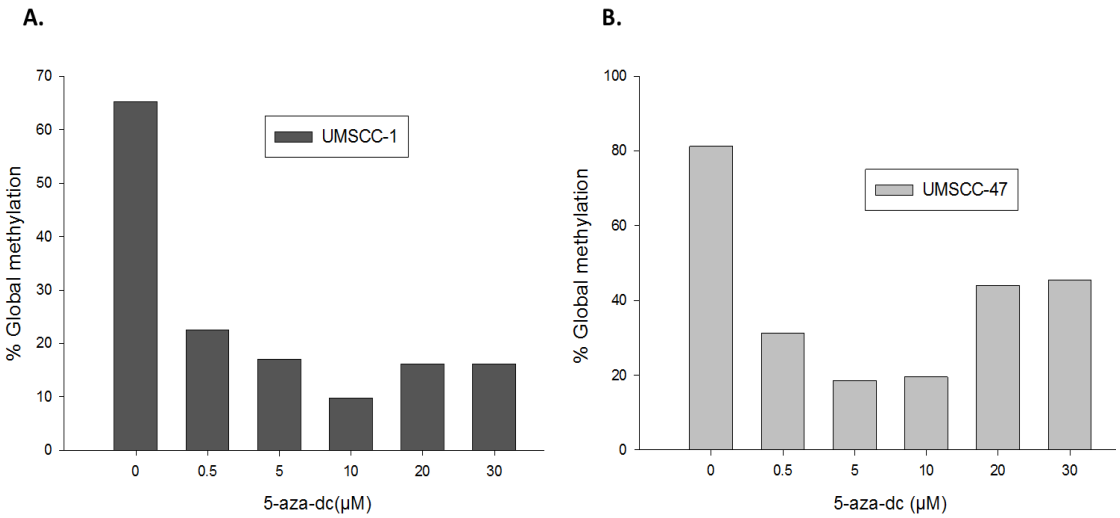

Figure 30: Changes in global methylation (%) in HPV-negative (UMSCC-1) cell line **(A)** and HPV-positive (UMSCC-47) cell line **(B)** after treatment with increasing dosage of 5-aza-dc for 96 hours.

Changes in expression of candidate tumor suppressor genes in de-methylation condition after 5-aza-dc treatment

To identify candidate genes whose expression is significantly increased upon de-methylation, HNC cell lines were treated with 5 μM 5-aza-dc for 96 hours and expression of the seven target genes was analyzed. As described in experimental procedures, the effect of treatment was statistically analyzed for each gene on each cell type individually as well as across the two cell types (*i.e.* Bonferroni corrected interaction), **Figure 31**. Out of 7 genes, 5 were less likely to be regulated specifically in HPV-positive HNCs. For example, *SMG1* was up-regulated upon de-methylation treatment only in HPV-negative UMSCC-1 cell line ($p=0.02$), but not in HPV-positive UMSCC-47 cell line ($p=0.795$). Although the significant change in expression of *CHFR, RUNX3,* and *RARB* was found on 5-aza-dc treatment, these alterations were observed in both cell lines. The expression of the fifth gene, *RUNX2* was unchanged in both cell lines on 5-aza-dc treatment.

With a goal to identify a gene that is epigenetically regulated specifically in the HPV-positive environment, I found two genes, *GRB7* and *EREG* that showed significant change on de-methylation as putative targets of HPV-mediated promoter methylation. De-methylation treatment significantly increased the expression of *GRB7* and *EREG* in HPV-positive UMSCC-47 cell line ($p<0.05$ and $p<0.001$ respectively) with no evidence of an effect in the HPV-negative UMSCC-1 cell line ($p=0.33$ and $p=0.21$ respectively), **Figure 31**. When the effect of 5-aza-dc treatment was analyzed across the two cell types (*i.e.* overall effect), I found no significant effect on *GRB7* and *EREG* expression across cell types (Bonferroni corrected interaction $p=0.26$ and $p=1.0$, respectively). This result further confirms that 5-aza-dc treatment has an effect on *GRB7* and *EREG* expression in cell-type specific manner but not in overall interaction across the cell lines.

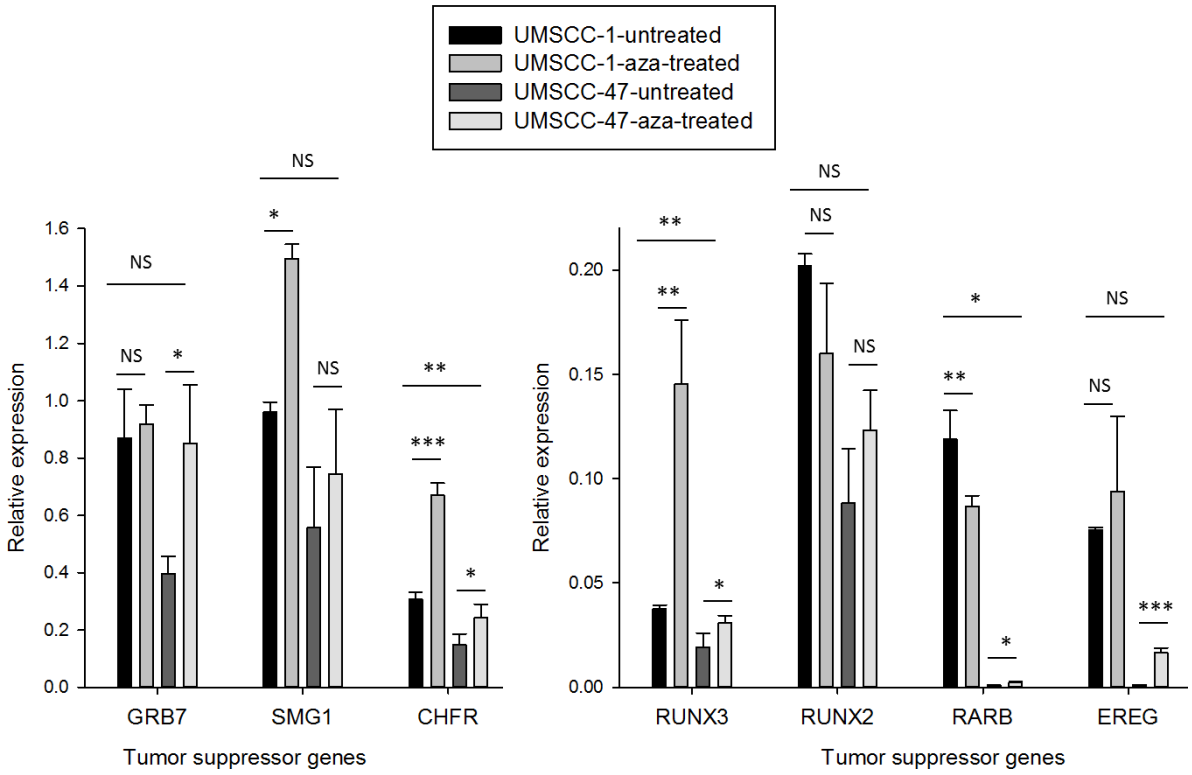

Figure 31: Changes in expression of tumor suppressor genes on de-methylation treatment (5 µM 5-aza-dc for 96 hours) in HPV-negative (UMSCC-1) and HPV-positive (UMSCC-47) cell lines. Statistical bars represents the changes on gene expressions on each cell line individually as well as Bonferroni corrected interaction of treatment across the two cell types (Significance level at *p<0.05, **p<0.01, **p<0.001 and NS non-significant)

EREG promoter was methylated in the HPV-positive HNC cell line and was hypomethylated after 5-aza-dc treatment

To further determine whether the silencing of *EREG* expression was due to promoter methylation, I examined the methylation status of the *EREG* promoter region in HPV-positive UMSCC-1 and HPV-negative UMSCC-47 cell lines using methylation-specific PCR (MS-PCR), which covered the CpG island in the promoter region of *EREG* (**Figure 32A**). MS-PCR assay revealed distinct differences in CpG methylation of the *EREG* promoter between UMSCC-1 and UMSCC-47 cell lines. A methylated band (lanes indicated by M) was identified in UMSCC-47 whereas an unmethylated band (lanes indicated by U) was observed in UMSCC-1 (**Figure 32B**). To further confirm methylation-mediated transcriptional silencing of *EREG*, cell lines were treated with a de-methylation agent (5-aza-dc). After 5 µM 5-aza-dc

treatment, the unmethylated band (U) was observed in UMSCC-47 cells, while no change was found in UMSCC-1 cells (**Figure 32C**). This suggests that the restoration of EREG mRNA expression following 5-aza-dc treatment in UMSCC-47 was likely caused by de-methylation of the hypermethylated CpG sites in the *EREG* promoter.

Methylation of GRB7 promoter in both HPV-positive and HPV-negative cell lines

Methylation-specific PCR analyses showed the presence of both methylated and unmethylated *GRB7* promoters in both cell lines, indicating partial methylation of *GRB7* in these cell types. Even on de-methylation treatment, there was no change in methylated and unmethylated bands on the agarose gel (**Figure 33B**). The promoter of *GRB7* contains CpG sites (**Figure 33A**) but does not seem to contain CpG island as predicted by MethPrimer software (Criteria used for CpG island prediction was Island size > 100 and GC% > 50.0). Therefore, I further checked for the presence of an upstream promoter, which can possibly affect the expression of *GRB7*. A study reported that methylation of *CSF3* promoter significantly correlates with *GRB7* expression; both *GRB7* and *CSF3* are on chromosome 17q and are commonly amplified together (218). Methylation-specific PCR of *CSF3* promoter also revealed similar results as observed from the *GRB7* promoter. Both methylated and unmethylated bands (lanes M and U respectively) of *CSF3* promoter were seen in UMSCC-1 and UMSCC-47 cell lines (**Figure 33D and 33C**). This indicates that epigenetic regulation of *GRB7* may not be specific to the HPV-positive environment. This result reduces enthusiasm for *GRB7* as a candidate gene for epigenetic regulation in HPV-associated HNCs.

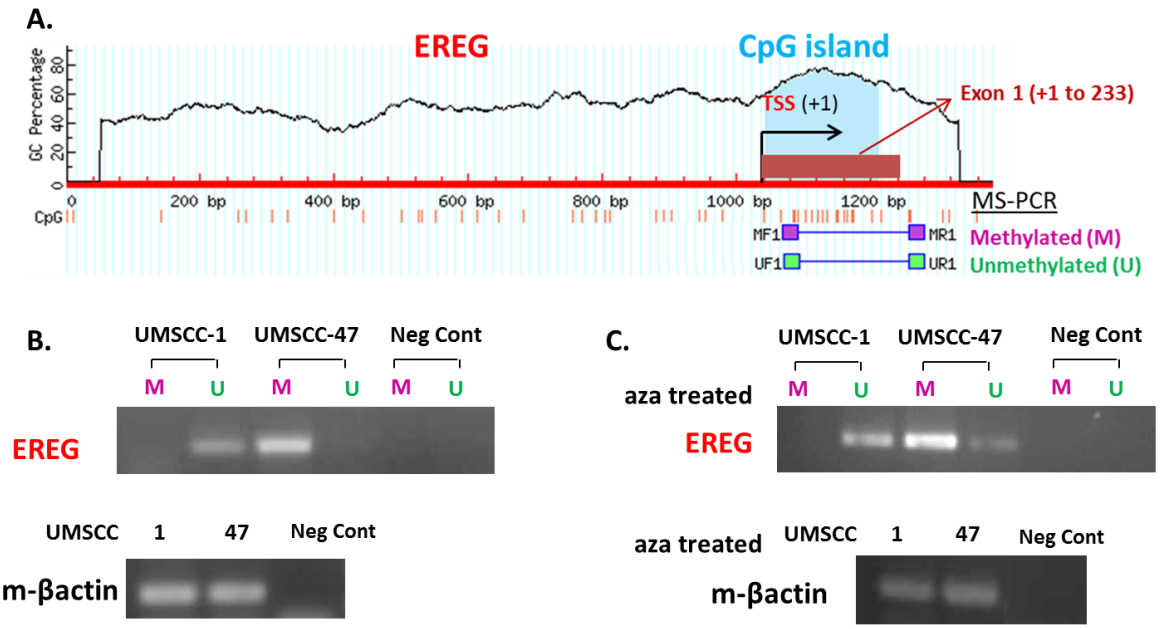

Figure 32: Methylation status of *EREG* promoter (**A)** *EREG* promoter showing CpG island. The locations where methylated and unmethylated methylation-specific PCR (MS-PCR) primers anneal are indicated by the rectangular box; MF1-MR1 (methylated- forward and reverse primers) and UF1-UR1 (unmethylated- forward and reverse primers). TSS indicates transcription start site. **(B)** MS-PCR of *EREG* in bisulfite-modified DNA from HPV-negative (UMSCC-1) and HPV-positive (UMSCC-47) cell lines. **(C)** MS-PCR of EREG after 5-aza-dc (5 µM for 96 hours) treatment. MS-PCR of β-actin serves as an internal control for bisulfite-modified DNA. No template DNA serves as negative control.

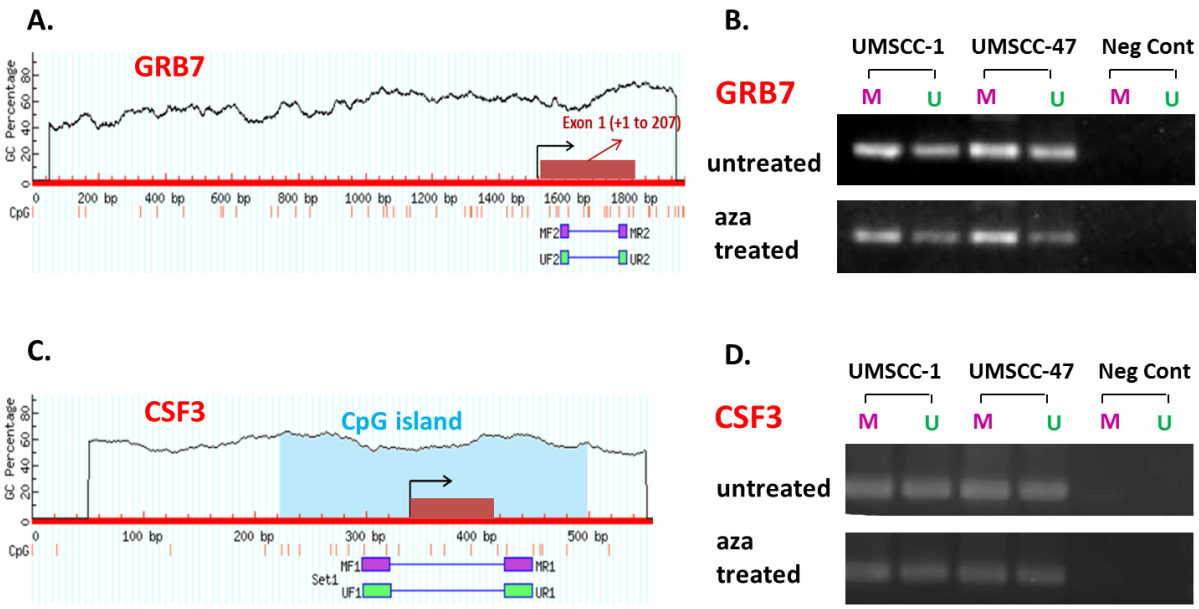

Figure 33: Methylation status of *GRB7* and *CSF3* promoters. **A)** *GRB7* promoter showing CpG sites and the locations of methylated and unmethylated methylation-specific PCR (MS-PCR) primers. MS-PCR of *GRB7* (**B)** and *CSF3* (**C** and **D)** in bisulfite-modified DNA from HPV-negative (UMSCC-1) and HPV-positive (UMSCC-47) cell lines with or without 5-aza-dc (5 µM for 96 hours) treatment. No template DNA serves as negative control. (Note: *CSF3* promoter methylation is known to regulate GRB7 expression)

Discussion

Epigenetic changes are considered as important mechanisms in HNC progression, but how these changes differ between HPV-positive and HPV-negative cancers are less defined. I conducted a study that helps to identify an epigenetic mechanism that is specific to HPV-positive HNCs. For this, I screened 38 tumor suppressor genes to identify a putative candidate regulated by promoter methylation.

From gene expression profile of 38 tumor suppressors in the custom array, I selected 7 genes (*GRB7*, *RUNX3*, *RUNX2*, *CHFR*, *RARB, EREG,* and *SMG-1*) as the targets for further studies, which showed reduced expressions in HPV-positive cell line than HPV-negative cell line (**Figure 28**). To determine whether the reduced expression of these genes is due to promoter methylation, I treated cell lines with a de-methylation agent, 5-aza-2′-deoxycytidine (5-aza-dc). It is shown that 5-aza-dc is able to re-express genes that are

silenced by DNA methylation; although, this reactivation can vary from gene to gene and different tissue/cell types (217). On 5-aza-dc treatment, I found different expression profiles of tumor suppressor genes in two HNC cell lines. Out of 7 genes, only *EREG* and *GRB7* genes expression was significantly increased upon de-methylation treatment specifically in HPV-positive UMSCC-47 cell line. Although *GRB7* expression was increased on 5-aza-dc treatment only in UMSCC-47, without any significant change in expression in UMSCC-1, *GRB7* promoter was found to be methylated in both cell lines. This indicates that epigenetic regulation of *GRB7* may not be specific to the HPV-positive environment. Further analyses quantifying the methylation status between UMSCC-1 and UMSCC-47 cell lines may help to better understand the relationship between gene methylation and transcriptional regulation in specific cell type.

In the case of *EREG*, I found a high correlation between gene expression and promoter methylation. Methylated *EREG* promoter was detected in UMSCC-47 (**Figure 32B**) where *EREG* was down-regulated (**Figure 28**). When these cells were treated with 5-aza-dc, *EREG* expression was restored (**Figure 31**) and the unmethylated band was seen (**Figure 32C**). In contrast, higher expression of *EREG* was found in the UMSCC-1 cell line (**Figure 28**), which corresponded with its unmethylated promoter (**Figure 32B**). Also, 5-aza-dc treatment showed no significant change in EREG expression in the UMSCC-1 cell line. Consequently, these results suggest that *EREG* is regulated via promoter methylation specifically in HPV-positive UMSCC-47 cell line. Subsequently, *EREG* was identified as a putative candidate gene for being a driver of the carcinogenic process specific to HPV-positive HNCs. Consistent with my results, a genome-wide methylation study done using two HPV-negative HNC cell lines (UMSCC-4 and UMSCC-74A) and an HPV-positive HNC cell line (UMSCC-47) have shown enrichment in *EREG* gene for hypermethylation and decreased expression in a UMSCC-47 (195). However, authors did not discuss the functional characterization of *EREG* in their study.

EREG gene encodes for Epiregulin protein, which is a member of the epidermal growth factor (EGF) family of proteins. Epiregulin was originally purified from the conditioned medium of the mouse fibroblast-derived tumor cell line and identified as a tumor growth

inhibitor, which induced morphological changes in HeLa cells (219). Epiregulin functions as one of the ligands for the ErbB family receptors, namely EGF receptor (EGFR or ErbB1) and ErbB4 (220). Studies suggested that epiregulin has dual biological activity (219,221,222). On one hand, it stimulates proliferation of fibroblasts, hepatocytes, smooth muscle cells, and keratinocytes (223-225) and *EREG* is overexpressed in many human cancers (226-228). On the other hand, *EREG* inhibits the growth of several epithelial tumor cells (219,221,229) and not all cancer cells have high levels of *EREG* expression (230). While the oncogenic function of epiregulin is known to involve the activation of PI3K/AKT and MAP kinase cell signaling pathways (228), little is known regarding the mechanism by which epiregulin functions as a tumor suppressor in epithelial cancers. It is suggested that epiregulin inhibits the binding of EGF (a potent mitogenic ligand of EGFR) to EGFR on epithelial tumor cells, which might be the reason for growth inhibition of these cells (219).

Little is known about the role of *EREG* in the development of HNCs. A study showed that epiregulin expression is higher in oral squamous cell carcinoma (OSCC) specimens than in normal gingiva and oral dysplasia (231), thereby indicating that epiregulin expression may be associated with cancer progression. Although authors did not determine HPV status of these OSCC specimens, I would expect mostly HPV-negative because my study and others (58,137) have shown that HPV prevalence is extremely low in OSCC. Another study done in two HPV-negative HNC cell lines showed that increased transcription of epiregulin was associated with the metastatic phenotype (232). Based on these findings, there is a high possibility that increased *EREG* expression is related to HPV-negative HNCs, but not to HPV-positive cancers. My study and Sartor *et. al* (195) showed the promoter methylation and transcriptional inactivation of *EREG* in HPV-positive HNC cell line, but not in HPV-negative HNC cell lines. This opens up a possibility that EREG may act as a tumor suppressor in HPV-positive HNC and as an oncogene in HPV-negative HNC. Further studies on HPV-positive and HPV-negative HNC cell lines and tissue specimens are required to confirm this hypothesis. Additionally, further studies have to be conducted to elucidate the role of *EREG* in HNC development.

Studying the role of *EREG* in head and neck carcinogenesis would be beneficial for the better application of treatment based on HPV status of a patient. Since HPV-positive HNC patients have better therapy response than HPV-negative HNC patients, clinical trials are undergoing to de-intensify the current treatment standards to reduce associated toxicities (173,233). So, de-intensification of chemotherapy has been implemented by replacing with cetuximab, a monoclonal antibody that inhibits EGFR. This is because *EGFR* is frequently overexpressed in HNCs independent of HPV etiology (180,181). Interestingly, some studies showed that the effect of cetuximab can be enhanced in cells with high levels of EGFR ligands such as EREG (221,230). In colorectal cancer, patients with high *EREG* expression benefit more from cetuximab therapy compared with low *EREG* expression (234). A study done in HNC cell lines also showed that expression of epiregulin (along with other EGFR ligands) is correlated with responsiveness to cetuximab (235). Because my study and Sartor *et. al* (195) showed that HPV presence in HNC cell lines causes decreased *EREG* expression, there is a possibility that HPV presence may decrease the efficacy of cetuximab therapy in HPV-positive HNCs. The further in-depth study is required to address this highly relevant issue and to determine whether 5-aza-dc induction of *EREG* expression increases sensitivity to cetuximab in HPV-positive HNCs.

There is a growing interest to use 5-aza-dc (also called decitabine) as a therapeutic agent for cancers in which epigenetic silencing of critical regulatory genes has occurred (217,236). Consequently, decitabine has been used in clinical trials for hematological and solid malignancies (237,238). The U.S. Food and Drug Administration (FDA) has approved decitabine for the treatment of myelodysplastic syndrome, a pre-leukemic bone marrow disorder (239). One group has investigated the efficacy of decitabine in combination with cisplatin in HNCs (240). However, studies are not yet done to explore the possibility of combinational therapy of a de-methylation agent decitabine and an EGFR-inhibitor cetuximab, which may be an effective therapeutic strategy for treating HNCs; particularly HPV-positive ones.

A major limitation of this study is that results were drawn based on two selected model cell lines representing HPV presence (UMSCC-47) and HPV absence (UMSCC-1) in HNCs. So, further studies are necessary using more HPV-positive/negative cell lines and

human HNC specimens to confirm whether *EREG* epigenetic regulation is specific to HPV-positive HNCs. Moreover, since HPV E7 is known to increase DNMT1 and DNMT3a activity (119), additional studies should be done by silencing E7 in HPV-positive cells or overexpressing E7 in HPV-negative cells to determine whether epigenetic inactivation of *EREG* is due to HPV E7 oncoprotein (see Appendix). Another limitation of this study is that the effect in gene expression using 5-aza-dc in combination with histone deacetylases (HDAC) inhibitor was not determined. Studies have indicated that 5-aza-dc in combination HDAC inhibitors can produce a synergetic reactivation of some genes (241,242), so future studies should be aimed using HDAC inhibitor alone or in combination with 5-aza-dc which would provide a better picture of the genes regulated by promoter methylation in HNCs.

Overall, in this chapter, I attempted to better characterize the methylation differences between HPV-positive and HPV-negative head and neck cancers. My finding concurs with the observation of Sartor *et. al* (195) that promoter hypermethylation may possibly contribute to down-regulation of *EREG* gene in the HPV-positive HNCs. This study should be continued to determine whether epigenetic inactivation of *EREG* plays a role in HPV-associated head and neck tumorigenesis.

CHAPTER VI

CONCLUDING REMARKS

My dissertation identifies the potential biomarkers and genetic and epigenetic mechanisms associated with HPV in head and neck tumors, as illustrated in **Figure 35**.

HPV-positive HNCs are characterized as a distinct entity from HNCs that are linked to tobacco and alcohol use, and lack HPV-related etiology. Of note, HPV-positive HNC patients are found to have more favorable treatment response and better survival than patients whose tumors are HPV-negative. As HPV detection has huge diagnostic and prognostic significance, there should be a suitable biomarker which precisely diagnoses HPV infection and a personalized treatment regimen based on HPV status. For these reasons, the knowledge of genetic and epigenetic profiles in HPV-positive HNCs is very important. This will help to distinguish the underlying molecular basis between HPV-associated and –unassociated carcinogenesis that may lead to the development of novel therapeutic strategies.

As the progression of HPV infection in premalignant lesions to HNCs is unknown, in-depth clinical, histologic and molecular characterization of HPV-associated head and neck tumors is needed. In chapter II, pathologically distinct tumors of the head and neck (benign, premalignant and malignant) were characterized both histologically and molecularly. I found that benign head and neck tumors *i.e.* multiple epithelial hyperplasias (MEH) are caused by low-risk HPV types (126), whereas premalignant and malignant head and neck tumors are caused by high-risk HPV types, predominantly HPV type 16 (127). Other benign lesions called oral squamous papillomas were mostly HPV negative. Additionally, I found that “mitosoid cells”, which were previously thought to represent an HPV-associated cytopathic effect in benign MEH, can also be found in HPV-positive premalignant head and neck tumors (127). My results strongly support that “mitosoid cells” in head and neck tumors are histologic biomarkers for HPV infection.

The identification of serum biomarkers of HPV infection is equally important, which could lead to the development of early and non-invasive methods of HPV detection. In chapter III, I assessed serum HPV E7 antibody titer as a potential serological biomarker. I have shown for the first time that the combination of HPV E7 seropositivity and $p16^{INK4a}$ expression is a strong diagnostic biomarker for HPV-associated head and neck malignancy (145). HPV E7 serology may be particularly useful in cases when a tissue specimen is not sufficient for histological tests (*e.g.* immunostaining of $p16^{INK4a}$ or detecting mitosoid cells). Further, serial monitoring of decline in HPV serology may be useful in predicting a patient's response to treatment.

In chapter IV, I extrapolated knowledge of HPV-induced oncogenicity in cervical cancer to understand molecular determinants of HPV-associated HNCs. I analyzed whether HPV DNA - integration and -methylation might play important roles in HPV-linked head and neck carcinogenesis as seen in HPV-associated cervical cancers. I found that integration of HPV DNA into the host genome appears to be essential for neoplastic transformation of premalignant lesions; however, HPV DNA methylation might not play a role in the process of HPV-induced head and neck carcinogenesis. I also suggest that Group 1 premalignant lesions which contain diffuse mitosoid cells might have higher malignant transforming potential, though further long-term follow-up studies are required to appropriately address any trends towards malignant transformation.

Study of HPV epigenetics indicated that viral DNA methylation might not be involved in head and neck carcinogenesis (Chapter IV). Therefore, in chapter V, I studied epigenetic changes (*i.e.* promoter methylation and transcriptional inactivation) of host genes specific to HPV- associated head and neck malignancy. For this, 38 tumor suppressor genes were selected based on literature and screened to identify a putative candidate. I found that HPV-positive HNC cell lines showed promoter hypermethylation and transcriptional inactivation of a candidate tumor suppressor gene *EREG*. It suggests that epigenetic regulation of *EREG* may be specific to HPV-positive HNCs and may play a role in the development of HPV-associated HNCs. This result needs to be put in a broader context using human cancer specimens and understanding the role of *EREG* in head and neck carcinogenesis. The *EREG* gene encodes

a ligand of the epidermal growth factor receptor (EGFR). Further studies on *EREG* gene could provide insights into possible treatment strategies using EGFR inhibitors (*i.e.* cetuximab, the Food and Drug Administration (FDA)-approved drug for HNC patients). Future studies are also needed to explore the possibility of using combinational therapy of two FDA-approved drugs - cetuximab and decitabine (5-aza-dc) for treating HNCs, particularly HPV-positive cancers.

Overall, in this dissertation, I found variation in HPV presence among pathologically distinct head and neck tumors. I also identified potential biomarkers of underlying HPV etiology in head and neck malignancy. This study also provides better insights into the putative genetic and epigenetic mechanisms by which HPV triggers head and neck carcinogenesis, though further studies are required to explore this highly relevant issue.

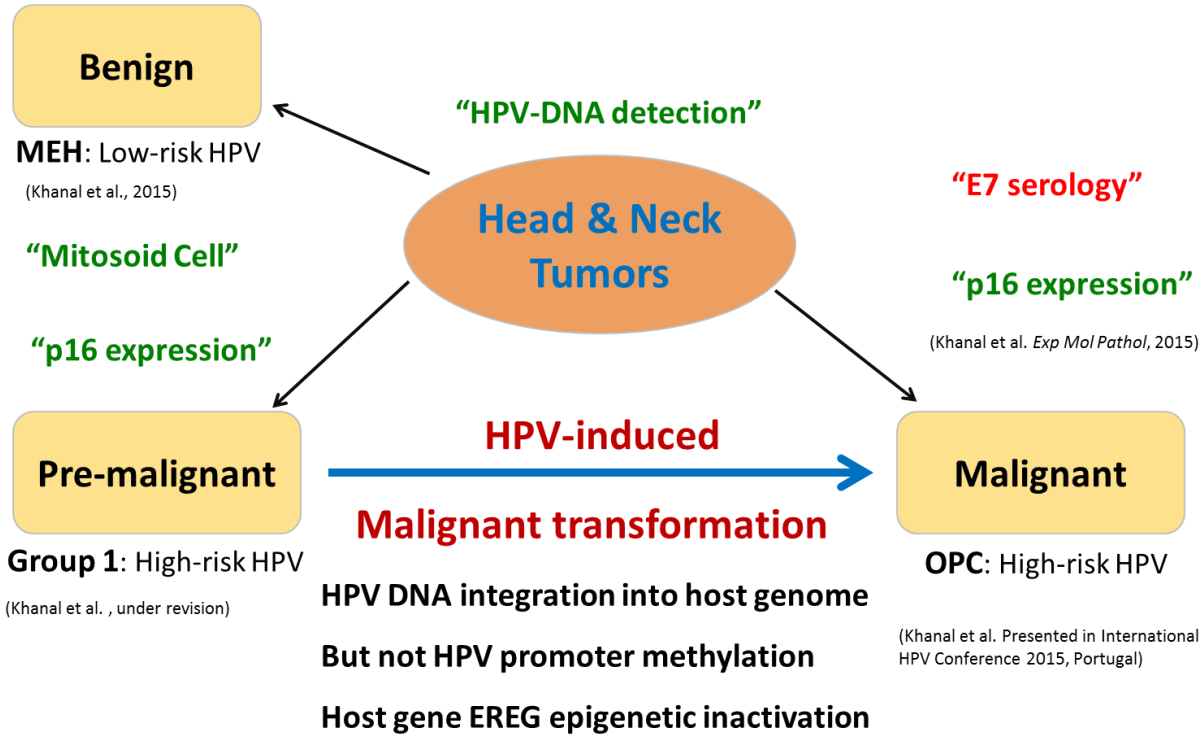

Figure 34: A schematic representation of a summary of the dissertation.

Different types of head and neck tumors were associated with the distinct type of HPV. Benign tumors, particularly multiple epithelial hyperplasias (MEH) were caused by low-risk HPV types. Pre-malignant (mainly Group1 lesions) and malignant (particularly oropharyngeal cancers, OPC) were caused by high-risk HPV types. Potential serum and tissue biomarkers for underlying HPV etiology were identified (shown in quotation marks). Mitosoid cells represented not only an HPV-associated cytopathic effect in benign MEH but also a subset of premalignant tumors. $p16^{INK4a}$ may serve as a surrogate marker only in Group 1 lesions compared to other premalignant lesions. E7 serology complements p16 expression as a strong biomarker for HPV-associated malignancy. Integration of HPV DNA and epigenetic inactivation of host gene *EREG* may play a role in HPV-associated malignancy. But, viral DNA methylation may not be required for transformation of premalignant lesions to malignant.

REFERENCES

1. de Villiers, E. M. (1989) Heterogeneity of the human papillomavirus group. *Journal of virology* **63**, 4898-4903
2. (2015) Human Papillomavirus (HPV). Available at http://www.cdc.gov/hpv/parents/questions-answers.html. Centers for Disease Control and Prevention (CDC). Last update: 12/28/2015; Accessed: 06/30/2016.
3. Forman, D., de Martel, C., Lacey, C. J., Soerjomataram, I., Lortet-Tieulent, J., Bruni, L., Vignat, J., Ferlay, J., Bray, F., and Plummer, M. (2012) Global burden of human papillomavirus and related diseases. *Vaccine* **30**, F12-F23
4. Powell, N. G., and Evans, M. (2015) Human papillomavirus-associated head and neck cancer: oncogenic mechanisms, epidemiology and clinical behaviour. *Diagnostic Histopathology* **21**, 49-64
5. Hausen, H. Z., Meinhof, W., Scheiber, W., and Bornkamm, G. W. (1974) Attempts to detect virus-specific DNA in human tumors. I. Nucleic acid hybridizations with complementary RNA of human wart virus. *International Journal of Cancer* **13**, 650-656
6. Dürst, M., Gissmann, L., Ikenberg, H., and Zur Hausen, H. (1983) A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. *Proceedings of the National Academy of Sciences* **80**, 3812-3815
7. Parkin, D. M. (2006) The global health burden of infection-associated cancers in the year 2002. *International journal of cancer* **118**, 3030-3044
8. García-Vallvé, S., Alonso, Á., and Bravo, I. G. (2005) Papillomaviruses: different genes have different histories. *Trends in microbiology* **13**, 514-521
9. Sakakibara, N., Mitra, R., and McBride, A. A. (2011) The papillomavirus E1 helicase activates a cellular DNA damage response in viral replication foci. *Journal of virology* **85**, 8981-8995
10. McBride, A. A. (2008) Replication and partitioning of papillomavirus genomes. *Advances in virus research* **72**, 155-205
11. Doorbar, J. (2013) The E4 protein; structure, function and patterns of expression. *Virology* **445**, 80-98
12. Rautava, J., and Syrjänen, S. (2012) Biology of human papillomavirus infections in head and neck carcinogenesis. *Head and neck pathology* **6**, 3-15
13. Venuti, A., Paolini, F., Nasir, L., Corteggio, A., Roperto, S., Campo, M. S., and Borzacchiello, G. (2011) Papillomavirus E5: the smallest oncoprotein with many functions. *Molecular cancer* **10**, 1
14. Klingelhutz, A. J., and Roman, A. (2012) Cellular transformation by human papillomaviruses: lessons learned by comparing high-and low-risk viruses. *Virology* **424**, 77-98
15. McLaughlin-Drubin, M. E., and Münger, K. (2009) Oncogenic activities of human papillomaviruses. *Virus research* **143**, 195-208
16. Fu, B., Quintero, J., and Baker, C. C. (2003) Keratinocyte growth conditions modulate telomerase expression, senescence, and immortalization by human papillomavirus type 16 E6 and E7 oncogenes. *Cancer research* **63**, 7815-7824

17. Xu, M., Katzenellenbogen, R. A., Grandori, C., and Galloway, D. A. (2013) An unbiased in vivo screen reveals multiple transcription factors that control HPV E6-regulated hTERT in keratinocytes. *Virology* **446**, 17-24
18. El-Naggar, A. K., and Westra, W. H. (2012) p16 expression as a surrogate marker for HPV-related oropharyngeal carcinoma: A guide for interpretative relevance and consistency. *Head & neck* **34**, 459-461
19. Romanczuk, H., Thierry, F., and Howley, P. (1990) Mutational analysis of cis elements involved in E2 modulation of human papillomavirus type 16 P97 and type 18 P105 promoters. *Journal of virology* **64**, 2849-2859
20. Thierry, F. (2009) Transcriptional regulation of the papillomavirus oncogenes by cellular and viral transcription factors in cervical carcinoma. *Virology* **384**, 375-379
21. Tan, S.-H., Leong, L., Walker, P. A., and Bernard, H.-U. (1994) The human papillomavirus type 16 E2 transcription factor binds with low cooperativity to two flanking sites and represses the E6 promoter through displacement of Sp1 and TFIID. *Journal of virology* **68**, 6411-6420
22. Arias-Pulido, H., Peyton, C. L., Joste, N. E., Vargas, H., and Wheeler, C. M. (2006) Human papillomavirus type 16 integration in cervical carcinoma in situ and in invasive cervical cancer. *Journal of clinical microbiology* **44**, 1755-1762
23. Clarke, M. A., Wentzensen, N., Mirabello, L., Ghosh, A., Wacholder, S., Harari, A., Lorincz, A., Schiffman, M., and Burk, R. D. (2012) Human papillomavirus DNA methylation as a potential biomarker for cervical cancer. *Cancer Epidemiology Biomarkers & Prevention* **21**, 2125-2137
24. Jacquin, E., Baraquin, A., Ramanah, R., Carcopino, X., Morel, A., Valmary-Degano, S., Bravo, I. G., de Sanjosé, S., Riethmuller, D., and Mougin, C. (2013) Methylation of Human Papillomavirus Type 16 CpG Sites at E2-Binding Site 1 (E2BS1), E2BS2, and the Sp1-Binding Site in Cervical Cancer Samples as Determined by High-Resolution Melting Analysis–PCR. *Journal of clinical microbiology* **51**, 3207-3215
25. Bernard, H.-U., Burk, R. D., Chen, Z., van Doorslaer, K., zur Hausen, H., and de Villiers, E.-M. (2010) Classification of papillomaviruses (PVs) based on 189 PV types and proposal of taxonomic amendments. *Virology* **401**, 70-79
26. De Villiers, E.-M., Fauquet, C., Broker, T. R., Bernard, H.-U., and zur Hausen, H. (2004) Classification of papillomaviruses. *Virology* **324**, 17-27
27. Cornet, I., Gheit, T., Iannacone, M., Vignat, J., Sylla, B., Del Mistro, A., Franceschi, S., Tommasino, M., and Clifford, G. (2013) HPV16 genetic variation and the development of cervical cancer worldwide. *British journal of cancer* **108**, 240-244
28. Ho, L., Chan, S. Y., Burk, R. D., Das, B., Fujinaga, K., Icenogle, J. P., Kahn, T., Kiviat, N., Lancaster, W., and Mavromara-Nazos, P. (1993) The genetic drift of human papillomavirus type 16 is a means of reconstructing prehistoric viral spread and the movement of ancient human populations. *Journal of virology* **67**, 6413-6423
29. Ho, L., Chan, S., Chow, V., Chong, T., Tay, S., Villa, L. L., and Bernard, H. (1991) Sequence variants of human papillomavirus type 16 in clinical samples permit verification and extension of epidemiological studies and construction of a phylogenetic tree. *Journal of clinical microbiology* **29**, 1765-1772
30. Munoz, N., Bosch, F. X., de Sanjose, S., Herrero, R., Castellsague, X., Shah, K. V., Snijders, P. J., Meijer, C. J., and International Agency for Research on Cancer Multicenter Cervical Cancer Study, G. (2003) Epidemiologic classification of human papillomavirus types associated with cervical cancer. *The New England journal of medicine* **348**, 518-527
31. Bouvard, V., Baan, R., Straif, K., Grosse, Y., Secretan, B., El Ghissassi, F., Benbrahim-Tallaa, L., Guha, N., Freeman, C., and Galichet, L. (2009) A review of human carcinogens—Part B: biological agents. *The lancet oncology* **10**, 321-322

32. Kreimer, A. R., Clifford, G. M., Boyle, P., and Franceschi, S. (2005) Human papillomavirus types in head and neck squamous cell carcinomas worldwide: a systematic review. *Cancer Epidemiology Biomarkers & Prevention* **14**, 467-475
33. Robinson, M., Sloan, P., and Shaw, R. (2010) Refining the diagnosis of oropharyngeal squamous cell carcinoma using human papillomavirus testing. *Oral oncology* **46**, 492-496
34. Bishop, J. A., Lewis, J. S., Jr., Rocco, J. W., and Faquin, W. C. (2015) HPV-related squamous cell carcinoma of the head and neck: An update on testing in routine pathology practice. *Seminars in diagnostic pathology* **32**, 344-351
35. Westra, W. H. (2014) Detection of human papillomavirus (HPV) in clinical samples: evolving methods and strategies for the accurate determination of HPV status of head and neck carcinomas. *Oral oncology* **50**, 771-779
36. Rooper, L. M., Gandhi, M., Bishop, J. A., and Westra, W. H. (2016) RNA in-situ hybridization is a practical and effective method for determining HPV status of oropharyngeal squamous cell carcinoma including discordant cases that are p16 positive by immunohistochemistry but HPV negative by DNA in-situ hybridization. *Oral Oncol.* **55**, 11-16
37. Bishop, J. A., Ma, X. J., Wang, H., Luo, Y., Illei, P. B., Begum, S., Taube, J. M., Koch, W. M., and Westra, W. H. (2012) Detection of transcriptionally active high-risk HPV in patients with head and neck squamous cell carcinoma as visualized by a novel E6/E7 mRNA in situ hybridization method. *Am. J. Surg. Pathol.* **36**, 1874-1882
38. Mirghani, H., Casiraghi, O., Amen, F., He, M., Ma, X. J., Saulnier, P., Lacroix, L., Drusch, F., Ben Lakdhar, A., Saint Guily, J. L., Badoual, C., Scoazec, J. Y., and Vielh, P. (2015) Diagnosis of HPV-driven head and neck cancer with a single test in routine clinical practice. *Mod. Pathol.* **28**, 1518-1527
39. Schache, A. G., Liloglou, T., Risk, J. M., Jones, T. M., Ma, X. J., Wang, H., Bui, S., Luo, Y., Sloan, P., Shaw, R. J., and Robinson, M. (2013) Validation of a novel diagnostic standard in HPV-positive oropharyngeal squamous cell carcinoma. *Br. J. Cancer* **108**, 1332-1339
40. Ukpo, O. C., Flanagan, J. J., Ma, X. J., Luo, Y., Thorstad, W. L., and Lewis, J. S., Jr. (2011) High-risk human papillomavirus E6/E7 mRNA detection by a novel in situ hybridization assay strongly correlates with p16 expression and patient outcomes in oropharyngeal squamous cell carcinoma. *Am. J. Surg. Pathol.* **35**, 1343-1350
41. Wang, F., Flanagan, J., Su, N., Wang, L. C., Bui, S., Nielson, A., Wu, X., Vo, H. T., Ma, X. J., and Luo, Y. (2012) RNAscope: a novel in situ RNA analysis platform for formalin-fixed, paraffin-embedded tissues. *The Journal of molecular diagnostics : JMD* **14**, 22-29
42. Morbini, P., Alberizzi, P., Tinelli, C., Paglino, C., Bertino, G., Comoli, P., Pedrazzoli, P., and Benazzo, M. (2015) Identification of transcriptionally active HPV infection in formalin-fixed, paraffin-embedded biopsies of oropharyngeal carcinoma. *Hum. Pathol.* **46**, 681-689
43. Vokes, E. E., Agrawal, N., and Seiwert, T. Y. (2015) HPV-Associated Head and Neck Cancer. *Journal of the National Cancer Institute* **107**, djv344
44. Giarre, M., Caldeira, S., Malanchi, I., Ciccolini, F., Leao, M. J., and Tommasino, M. (2001) Induction of pRb degradation by the human papillomavirus type 16 E7 protein is essential to efficiently overcome p16INK4a-imposed G1 cell cycle Arrest. *Journal of virology* **75**, 4705-4712
45. Zur Hausen, H. (2002) Papillomaviruses and cancer: from basic studies to clinical application. *Nature Reviews Cancer* **2**, 342-350

46. Wang, H., Sun, R., Lin, H., and Hu, W. h. (2013) P16INK4A as a surrogate biomarker for human papillomavirus-associated oropharyngeal carcinoma: Consideration of some aspects. *Cancer science* **104**, 1553-1559
47. Westra, W. H. (2015) The pathology of HPV-related head and neck cancer: implications for the diagnostic pathologist. *Seminars in diagnostic pathology* **32**, 42-53
48. Smith, E. M., Wang, D., Kim, Y., Rubenstein, L. M., Lee, J. H., Haugen, T. H., and Turek, L. P. (2008) P16 INK4a expression, human papillomavirus, and survival in head and neck cancer. *Oral oncology* **44**, 133-142
49. Said, A. K., Leao, J. C., Fedele, S., and Porter, S. R. (2013) Focal epithelial hyperplasia–an update. *Journal of Oral Pathology & Medicine* **42**, 435-442
50. Syrjänen, S. (2003) Human papillomavirus infections and oral tumors. *Medical microbiology and immunology* **192**, 123-128
51. Garlick, J. A., Calderon, S., Buchner, A., and Mitrani-Rosenbaum, S. (1989) Detection of human papillomavirus (HPV) DNA in focal epithelial hyperplasia. *Journal of Oral Pathology & Medicine* **18**, 172-177
52. Syrjänen, K. J., and Syrjänen, S. M. (2000) *Papillomavirus infections in human pathology*, Wiley
53. Zeuss, M. S., Miller, C. S., and White, D. K. (1991) In situ hybridization analysis of human papillomavirus DNA in oral mucosal lesions. *Oral surgery, oral medicine, oral pathology* **71**, 714-720
54. Castro, T. P., and Bussoloti Filho, I. (2006) Prevalence of human papillomavirus (HPV) in oral cavity and oropharynx. *Brazilian journal of otorhinolaryngology* **72**, 272-282
55. Eversole, L., and Laipis, P. (1988) Oral squamous papillomas: detection of HPV DNA by in situ hybridization. *Oral surgery, oral medicine, oral pathology* **65**, 545-550
56. Feller, L., Khammissa, R., Wood, N., Marnewick, J., Meyerov, R., and Lemmer, J. (2011) HPV-associated oral warts. *SADJ: journal of the South African Dental Association= tydskrif van die Suid-Afrikaanse Tandheelkundige Vereniging* **66**, 82-85
57. Pai, S. I., and Westra, W. H. (2009) Molecular pathology of head and neck cancer: implications for diagnosis, prognosis, and treatment. *Annual review of pathology* **4**, 49
58. Lingen, M. W., Xiao, W., Schmitt, A., Jiang, B., Pickard, R., Kreinbrink, P., Perez-Ordonez, B., Jordan, R. C., and Gillison, M. L. (2013) Low etiologic fraction for high-risk human papillomavirus in oral cavity squamous cell carcinomas. *Oral oncology* **49**, 1-8
59. Woodman, C. B., Collins, S. I., and Young, L. S. (2007) The natural history of cervical HPV infection: unresolved issues. *Nature reviews. Cancer* **7**, 11-22
60. Parkin, D. M., Bray, F., Ferlay, J., and Pisani, P. (2005) Global cancer statistics, 2002. *CA: a cancer journal for clinicians* **55**, 74-108
61. Boscolo-Rizzo, P., Del Mistro, A., Bussu, F., Lupato, V., Baboci, L., Almadori, G., MC, D. A. M., and Paludetti, G. (2013) New insights into human papillomavirus-associated head and neck squamous cell carcinoma. *Acta otorhinolaryngologica Italica : organo ufficiale della Societa italiana di otorinolaringologia e chirurgia cervico-facciale* **33**, 77-87
62. Chaturvedi, A. K., Engels, E. A., Pfeiffer, R. M., Hernandez, B. Y., Xiao, W., Kim, E., Jiang, B., Goodman, M. T., Sibug-Saber, M., Cozen, W., Liu, L., Lynch, C. F., Wentzensen, N., Jordan, R. C., Altekruse, S., Anderson, W. F., Rosenberg, P. S., and Gillison, M. L. (2011) Human papillomavirus and rising oropharyngeal cancer incidence in the United States. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **29**, 4294-4301

63. Fakhry, C., Westra, W. H., Li, S., Cmelak, A., Ridge, J. A., Pinto, H., Forastiere, A., and Gillison, M. L. (2008) Improved survival of patients with human papillomavirus-positive head and neck squamous cell carcinoma in a prospective clinical trial. *Journal of the National Cancer Institute* **100**, 261-269
64. Chen, S. F., Yu, F. S., Chang, Y. C., Fu, E., Nieh, S., and Lin, Y. S. (2012) Role of human papillomavirus infection in carcinogenesis of oral squamous cell carcinoma with evidences of prognostic association. *Journal of oral pathology & medicine : official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology* **41**, 9-15
65. Owadally, W., Hurt, C., Timmins, H., Parsons, E., Townsend, S., Patterson, J., Hutcheson, K., Powell, N., Beasley, M., and Palaniappan, N. (2015) PATHOS: a phase II/III trial of risk-stratified, reduced intensity adjuvant treatment in patients undergoing transoral surgery for Human papillomavirus (HPV) positive oropharyngeal cancer. *BMC cancer* **15**, 1
66. Kimple, R. J., and Harari, P. M. (2014) Is radiation dose reduction the right answer for HPV-positive head and neck cancer? *Oral oncology* **50**, 560-564
67. Agrawal, N., Frederick, M. J., Pickering, C. R., Bettegowda, C., Chang, K., Li, R. J., Fakhry, C., Xie, T. X., Zhang, J., Wang, J., Zhang, N., El-Naggar, A. K., Jasser, S. A., Weinstein, J. N., Trevino, L., Drummond, J. A., Muzny, D. M., Wu, Y., Wood, L. D., Hruban, R. H., Westra, W. H., Koch, W. M., Califano, J. A., Gibbs, R. A., Sidransky, D., Vogelstein, B., Velculescu, V. E., Papadopoulos, N., Wheeler, D. A., Kinzler, K. W., and Myers, J. N. (2011) Exome sequencing of head and neck squamous cell carcinoma reveals inactivating mutations in NOTCH1. *Science* **333**, 1154-1157
68. Nichols, A. C., Chan-Seng-Yue, M., Yoo, J., Xu, W., Dhaliwal, S., Basmaji, J., Szeto, C. C., Dowthwaite, S., Todorovic, B., Starmans, M. H., Lambin, P., Palma, D. A., Fung, K., Franklin, J. H., Wehrli, B., Kwan, K., Koropatnick, J., Mymryk, J. S., Boutros, P., and Barrett, J. W. (2012) A Pilot Study Comparing HPV-Positive and HPV-Negative Head and Neck Squamous Cell Carcinomas by Whole Exome Sequencing. *ISRN oncology* **2012**, 809370
69. (2013) National Cancer Institute-Head and Neck Cancers. U.S. Department of Health and Human Services, National Institutes of Health National Cancer Institute, USA.gov, http://www.cancer.gov/types/head-and-neck/head-neck-fact-sheet
70. Sturgis, E. M., and Ang, K. K. (2011) The epidemic of HPV-associated oropharyngeal cancer is here: is it time to change our treatment paradigms? *Journal of the National Comprehensive Cancer Network : JNCCN* **9**, 665-673
71. O'Sullivan, B., Huang, S. H., Su, J., Garden, A. S., Sturgis, E. M., Dahlstrom, K., Lee, N., Riaz, N., Pei, X., Koyfman, S. A., Adelstein, D., Burkey, B. B., Friborg, J., Kristensen, C. A., Gothelf, A. B., Hoebers, F., Kremer, B., Speel, E. J., Bowles, D. W., Raben, D., Karam, S. D., Yu, E., and Xu, W. (2016) Development and validation of a staging system for HPV-related oropharyngeal cancer by the International Collaboration on Oropharyngeal cancer Network for Staging (ICON-S): a multicentre cohort study. *The Lancet. Oncology*
72. Cohan, D. M., Popat, S., Kaplan, S. E., Rigual, N., Loree, T., and Hicks, W. L., Jr. (2009) Oropharyngeal cancer: current understanding and management. *Current opinion in otolaryngology & head and neck surgery* **17**, 88-94
73. Harris, S. L., Thorne, L. B., Seaman, W. T., Neil Hayes, D., Couch, M. E., and Kimple, R. J. (2011) Association of p16INK4a overexpression with improved outcomes in young patients with squamous cell cancers of the oral tongue. *Head & neck* **33**, 1622-1627
74. Mirghani, H., Amen, F., Moreau, F., and St Guily, J. L. (2015) Do high-risk human papillomaviruses cause oral cavity squamous cell carcinoma? *Oral oncology* **51**, 229-236

75. El-Mofty, S. K., and Lu, D. W. (2003) Prevalence of human papillomavirus type 16 DNA in squamous cell carcinoma of the palatine tonsil, and not the oral cavity, in young patients: a distinct clinicopathologic and molecular disease entity. *The American journal of surgical pathology* **27**, 1463-1470
76. Poling, J., Ma, X.-J., Bui, S., Luo, Y., Li, R., Koch, W., and Westra, W. (2014) Human papillomavirus (HPV) status of non-tobacco related squamous cell carcinomas of the lateral tongue. *Oral oncology* **50**, 306-310
77. Isayeva, T., Li, Y., Maswahu, D., and Brandwein-Gensler, M. (2012) Human papillomavirus in non-oropharyngeal head and neck cancers: a systematic literature review. *Head and neck pathology* **6**, 104-120
78. Münger, K., Baldwin, A., Edwards, K. M., Hayakawa, H., Nguyen, C. L., Owens, M., Grace, M., and Huh, K. (2004) Mechanisms of human papillomavirus-induced oncogenesis. *Journal of virology* **78**, 11451-11460
79. Doorbar, J. (2006) Molecular biology of human papillomavirus infection and cervical cancer. *Clinical science* **110**, 525-541
80. Doorbar, J., Egawa, N., Griffin, H., Kranjec, C., and Murakami, I. (2015) Human papillomavirus molecular biology and disease association. *Reviews in medical virology* **25**, 2-23
81. Jenkins, D. (2007) Histopathology and cytopathology of cervical cancer. *Disease markers* **23**, 199
82. Syrjänen, S. (2004) HPV infections and tonsillar carcinoma. *Journal of clinical pathology* **57**, 449-455
83. Kim, S. H., Koo, B. S., Kang, S., Park, K., Kim, H., Lee, K. R., Lee, M. J., Kim, J. M., Choi, E. C., and Cho, N. H. (2007) HPV integration begins in the tonsillar crypt and leads to the alteration of p16, EGFR and c-myc during tumor formation. *International Journal of Cancer* **120**, 1418-1425
84. Chi, A. C., Day, T. A., and Neville, B. W. (2015) Oral cavity and oropharyngeal squamous cell carcinoma—an update. *CA: a cancer journal for clinicians* **65**, 401-421
85. Gupta, A. K., and Kumar, M. (2015) HPVbase–a knowledgebase of viral integrations, methylation patterns and microRNAs aberrant expression: As potential biomarkers for Human papillomaviruses mediated carcinomas. *Scientific reports* **5**
86. Sova, P., Feng, Q., Geiss, G., Wood, T., Strauss, R., Rudolf, V., Lieber, A., and Kiviat, N. (2006) Discovery of novel methylation biomarkers in cervical carcinoma by global demethylation and microarray analysis. *Cancer Epidemiology Biomarkers & Prevention* **15**, 114-123
87. Woo, H. J., Kim, S. J., Song, K.-J., Kim, S. S., Yoon, C.-H., Choi, B.-S., and Rhee, J. E. (2015) Hypermethylation of the tumor-suppressor cell adhesion molecule 1 in human papillomavirus-transformed cervical carcinoma cells. *International journal of oncology* **46**, 2656-2662
88. Narayan, G., Arias-Pulido, H., Koul, S., Vargas, H., Zhang, F. F., Villella, J., Schneider, A., Terry, M. B., Mansukhani, M., and Murty, V. V. (2003) Frequent promoter methylation of CDH1, DAPK, RARB, and HIC1 genes in carcinoma of cervix uteri: its relationship to clinical outcome. *Molecular cancer* **2**, 1
89. Zhang, Y., Chen, F., Sun, Y., Zhou, S., Li, T., and Chen, R. (2011) Effects of DNMT1 silencing on malignant phenotype and methylated gene expression in cervical cancer cells. *J Exp Clin Cancer Res* **30**, 98
90. Kalantari, M., Calleja-Macias, I. E., Tewari, D., Hagmar, B., Lie, K., Barrera-Saldana, H. A., Wiley, D. J., and Bernard, H.-U. (2004) Conserved methylation patterns of human papillomavirus type 16 DNA in asymptomatic infection and cervical neoplasia. *Journal of virology* **78**, 12762-12772

91. Williams, V. M., Filippova, M., Soto, U., and Duerksen-Hughes, P. J. (2011) HPV-DNA integration and carcinogenesis: putative roles for inflammation and oxidative stress. *Future virology* **6**, 45-57
92. Hopman, A. H., Smedts, F., Dignef, W., Ummelen, M., Sonke, G., Mravunac, M., Vooijs, G. P., Speel, E. J. M., and Ramaekers, F. (2004) Transition of high-grade cervical intraepithelial neoplasia to micro-invasive carcinoma is characterized by integration of HPV 16/18 and numerical chromosome abnormalities. *The Journal of pathology* **202**, 23-33
93. Hudelist, G., Manavi, M., Pischinger, K. I., Watkins-Riedel, T., Singer, C. F., Kubista, E., and Czerwenka, K. F. (2004) Physical state and expression of HPV DNA in benign and dysplastic cervical tissue: different levels of viral integration are correlated with lesion grade. *Gynecol Oncol* **92**, 873-880
94. Vinokurova, S., Wentzensen, N., Kraus, I., Klaes, R., Driesch, C., Melsheimer, P., Kisseljov, F., Durst, M., Schneider, A., and von Knebel Doeberitz, M. (2008) Type-dependent integration frequency of human papillomavirus genomes in cervical lesions. *Cancer research* **68**, 307-313
95. Ziegert, C., Wentzensen, N., Vinokurova, S., Kisseljov, F., Einenkel, J., Hoeckel, M., and von Knebel Doeberitz, M. (2003) A comprehensive analysis of HPV integration loci in anogenital lesions combining transcript and genome-based amplification techniques. *Oncogene* **22**, 3977-3984
96. Andersson, S., Safari, H., Mints, M., Lewensohn-Fuchs, I., Gyllensten, U., and Johansson, B. (2005) Type distribution, viral load and integration status of high-risk human papillomaviruses in pre-stages of cervical cancer (CIN). *British journal of cancer* **92**, 2195-2200
97. Badaracco, G., and Venuti, A. (2005) Physical status of HPV types 16 and 18 in topographically different areas of genital tumours and in paired tumour-free mucosa. *International journal of oncology* **27**, 161-167
98. Shin, H.-J., Joo, J., Yoon, J. H., Yoo, C. W., and Kim, J.-Y. (2014) Physical status of human papillomavirus integration in cervical cancer is associated with treatment outcome of the patients treated with radiotherapy. *PloS one* **9**, e78995
99. Tornesello, M. L., Buonaguro, L., Giorgi-Rossi, P., and Buonaguro, F. M. (2013) Viral and cellular biomarkers in the diagnosis of cervical intraepithelial neoplasia and cancer. *BioMed research international* **2013**
100. Olthof, N. C., Speel, E.-J. M., Kolligs, J., Haesevoets, A., Henfling, M., Ramaekers, F. C., Preuss, S. F., Drebber, U., Wieland, U., and Silling, S. (2014) Comprehensive analysis of HPV16 integration in OSCC reveals no significant impact of physical status on viral oncogene and virally disrupted human gene expression. *PloS one* **9**, e88718
101. Lace, M. J., Anson, J. R., Klussmann, J. P., Wang, D. H., Smith, E. M., Haugen, T. H., and Turek, L. P. (2011) Human papillomavirus type 16 (HPV-16) genomes integrated in head and neck cancers and in HPV-16-immortalized human keratinocyte clones express chimeric virus-cell mRNAs similar to those found in cervical cancers. *Journal of virology* **85**, 1645-1654
102. Ragin, C. C. R., Reshmi, S. C., and Gollin, S. M. (2004) Mapping and analysis of HPV16 integration sites in a head and neck cancer cell line. *International journal of cancer* **110**, 701-709
103. Hafkamp, H. C., Speel, E. J., Haesevoets, A., Bot, F. J., Dinjens, W. N., Ramaekers, F., Hopman, A. H., and Manni, J. J. (2003) A subset of head and neck squamous cell carcinomas exhibits integration of HPV 16/18 DNA and overexpression of p16INK4A and p53 in the absence of mutations in p53 exons 5–8. *International journal of cancer* **107**, 394-400

104. Parfenov, M., Pedamallu, C. S., Gehlenborg, N., Freeman, S. S., Danilova, L., Bristow, C. A., Lee, S., Hadjipanayis, A. G., Ivanova, E. V., and Wilkerson, M. D. (2014) Characterization of HPV and host genome interactions in primary head and neck cancers. *Proceedings of the National Academy of Sciences* **111**, 15544-15549
105. Begum, S., Cao, D., Gillison, M., Zahurak, M., and Westra, W. H. (2005) Tissue distribution of human papillomavirus 16 DNA integration in patients with tonsillar carcinoma. *Clinical Cancer Research* **11**, 5694-5699
106. Wiest, T., Schwarz, E., Enders, C., Flechtenmacher, C., and Bosch, F. X. (2002) Involvement of intact HPV16 E6/E7 gene expression in head and neck cancers with unaltered p53 status and perturbed pRb cell cycle control. *Oncogene* **21**, 1510-1517
107. Gao, G., Johnson, S. H., Kasperbauer, J. L., Eckloff, B. W., Tombers, N. M., Vasmatzis, G., and Smith, D. I. (2014) Mate pair sequencing of oropharyngeal squamous cell carcinomas reveals that HPV integration occurs much less frequently than in cervical cancer. *Journal of Clinical Virology* **59**, 195-200
108. Mellin, H., Dahlgren, L., Munck-Wikland, E., Lindholm, J., Rabbani, H., Kalantari, M., and Dalianis, T. (2002) Human papillomavirus type 16 is episomal and a high viral load may be correlated to better prognosis in tonsillar cancer. *International journal of cancer* **102**, 152-158
109. Sun, C., Reimers, L. L., and Burk, R. D. (2011) Methylation of HPV16 genome CpG sites is associated with cervix precancer and cancer. *Gynecologic oncology* **121**, 59-63
110. Mirabello, L., Schiffman, M., Ghosh, A., Rodriguez, A. C., Vasiljevic, N., Wentzensen, N., Herrero, R., Hildesheim, A., Wacholder, S., and Scibior-Bentkowska, D. (2013) Elevated methylation of HPV16 DNA is associated with the development of high grade cervical intraepithelial neoplasia. *International Journal of Cancer* **132**, 1412-1422
111. Wilson, G. A., Lechner, M., Köferle, A., Caren, H., Butcher, L. M., Feber, A., Fenton, T., Jay, A., Boshoff, C., and Beck, S. (2013) Integrated virus-host methylome analysis in head and neck squamous cell carcinoma. *Epigenetics* **8**, 953-961
112. Park, I.-S., Chang, X., Loyo, M., Wu, G., Chuang, A., Kim, M. S., Chae, Y. K., Lyford-Pike, S., Westra, W. H., and Saunders, J. R. (2011) Characterization of the methylation patterns in human papillomavirus type 16 viral DNA in head and neck cancers. *Cancer Prevention Research* **4**, 207-217
113. Kulis, M., and Esteller, M. (2010) DNA methylation and cancer. *Advances in genetics* **70**, 27-56
114. Baylin, S. B., Esteller, M., Rountree, M. R., Bachman, K. E., Schuebel, K., and Herman, J. G. (2001) Aberrant patterns of DNA methylation, chromatin formation and gene expression in cancer. *Human molecular genetics* **10**, 687-692
115. Robertson, K. D. (2001) DNA methylation, methyltransferases, and cancer. *Oncogene* **20**
116. Sharma, S., Kelly, T. K., and Jones, P. A. (2010) Epigenetics in cancer. *Carcinogenesis* **31**, 27-36
117. Takai, N., and Narahara, H. (2008) Array-based approaches for the identification of epigenetic silenced tumor suppressor genes. *Current genomics* **9**, 22-24
118. Costello, J. F., Frühwald, M. C., Smiraglia, D. J., Rush, L. J., Robertson, G. P., Gao, X., Wright, F. A., Feramisco, J. D., Peltomäki, P., and Lang, J. C. (2000) Aberrant CpG-island methylation has non-random and tumour-type–specific patterns. *Nature genetics* **24**, 132-138
119. Burgers, W., Blanchon, L., Pradhan, S., De Launoit, Y., Kouzarides, T., and Fuks, F. (2007) Viral oncoproteins target the DNA methyltransferases. *Oncogene* **26**, 1650-1655

120. Chalertpet, K., Pakdeechaidan, W., Patel, V., Mutirangura, A., and Yanatatsaneejit, P. (2015) Human papillomavirus type 16 E7 oncoprotein mediates CCNA1 promoter methylation. *Cancer science* **106**, 1333-1340
121. Bennett, K. L., Karpenko, M., Lin, M.-t., Claus, R., Arab, K., Dyckhoff, G., Plinkert, P., Herpel, E., Smiraglia, D., and Plass, C. (2008) Frequently methylated tumor suppressor genes in head and neck squamous cell carcinoma. *Cancer research* **68**, 4494-4499
122. Worsham, M. J., Chen, K. M., Stephen, J. K., Havard, S., and Benninger, M. S. (2010) Novel approaches to global mining of aberrantly methylated promoter sites in squamous head and neck cancer. *Otolaryngology-Head and Neck Surgery* **143**, 116-121. e119
123. Bennett, K. L., Lee, W., Lamarre, E., Zhang, X., Seth, R., Scharpf, J., Hunt, J., and Eng, C. (2010) HPV status-independent association of alcohol and tobacco exposure or prior radiation therapy with promoter methylation of FUSSEL18, EBF3, IRX1, and SEPT9, but not SLC5A8, in head and neck squamous cell carcinomas. *Genes, Chromosomes and Cancer* **49**, 319-326
124. Demokan, S., and Dalay, N. (2011) Role of DNA methylation in head and neck cancer. *Clinical epigenetics* **2**, 123-150
125. Guerrero-Preston, R., Michailidi, C., Marchionni, L., Pickering, C. R., Frederick, M. J., Myers, J. N., Yegnasubramanian, S., Hadar, T., Noordhuis, M. G., and Zizkova, V. (2014) Key tumor suppressor genes inactivated by "greater promoter" methylation and somatic mutations in head and neck cancer. *Epigenetics* **9**, 1031-1046
126. Khanal, S., Cole, E. T., Joh, J., Ghim, S. J., Jenson, A. B., Rai, S. N., Trainor, P. J., and Shumway, B. S. (2015) Human papillomavirus detection in histologic samples of multifocal epithelial hyperplasia: a novel demographic presentation. *Oral surgery, oral medicine, oral pathology and oral radiology* **120**, 733-743
127. Shumway, B., Khanal, S., Trainor, P., Zahin, M., Ghim, S., Joh, J., Rai, S., and Jenson, A. (2016) Histologic Variation in High-Grade Oral Epithelial Dysplasia When Associated With High-risk Human Papillomavirus. *International journal of radiation oncology, biology, physics* **4**, 944
128. Walker, D. (1998) Histological Typing of Cancer and Precancer of the Oral Mucosa. *Pathology* **30**, 87
129. Bussu, F., Sali, M., Gallus, R., Vellone, V. G., Zannoni, G., Autorino, R., Dinapoli, N., Santangelo, R., Martucci, R., and Graziani, C. (2013) HPV infection in squamous cell carcinomas arising from different mucosal sites of the head and neck region. Is p16 immunohistochemistry a reliable surrogate marker&quest. *British journal of cancer* **108**, 1157-1162
130. Jayaprakash, V., Reid, M., Hatton, E., Merzianu, M., Rigual, N., Marshall, J., Gill, S., Frustino, J., Wilding, G., and Loree, T. (2011) Human papillomavirus types 16 and 18 in epithelial dysplasia of oral cavity and oropharynx: a meta-analysis, 1985–2010. *Oral oncology* **47**, 1048-1054
131. Woo, S.-B., Cashman, E. C., and Lerman, M. A. (2013) Human papillomavirus-associated oral intraepithelial neoplasia. *Modern Pathology* **26**, 1288-1297
132. McCord, C., Xu, J., Xu, W., Qiu, X., McComb, R. J., Perez-Ordonez, B., and Bradley, G. (2013) Association of high-risk human papillomavirus infection with oral epithelial dysplasia. *Oral surgery, oral medicine, oral pathology and oral radiology* **115**, 541-549
133. (2014) HPV-Associated Oropharyngeal Cancer Rates by Race and Ethnicity. Available at http://www.cdc.gov/cancer/hpv/statistics/headneck.htm. Centers for Disease Control and Prevention (CDC). Last update: 06/05/2014; Accessed: 06/30/2016.

134. Organization, W. H. (2004) *International statistical classification of diseases and related health problems*, World Health Organization
135. Hashibe, M., Brennan, P., Benhamou, S., Castellsague, X., Chen, C., Curado, M. P., Dal Maso, L., Daudt, A. W., Fabianova, E., and Wünsch-Filho, V. (2007) Alcohol drinking in never users of tobacco, cigarette smoking in never drinkers, and the risk of head and neck cancer: pooled analysis in the International Head and Neck Cancer Epidemiology Consortium. *Journal of the National Cancer Institute* **99**, 777-789
136. Paleri, V., Mehanna, H., and Wight, R. (2010) EDITORIAL: TNM classification of malignant tumours 7th edition: what's new for head and neck? *Clinical Otolaryngology* **35**, 270-272
137. Chaturvedi, A. K., Engels, E. A., Anderson, W. F., and Gillison, M. L. (2008) Incidence trends for human papillomavirus–related and–unrelated oral squamous cell carcinomas in the United States. *Journal of Clinical Oncology* **26**, 612-619
138. Gillison, M. L., D'Souza, G., Westra, W., Sugar, E., Xiao, W., Begum, S., and Viscidi, R. (2008) Distinct risk factor profiles for human papillomavirus type 16–positive and human papillomavirus type 16–negative head and neck cancers. *Journal of the National Cancer Institute* **100**, 407-420
139. Allen, C. T., Lewis, J. S., El-Mofty, S. K., Haughey, B. H., and Nussenbaum, B. (2010) Human papillomavirus and oropharynx cancer: biology, detection and clinical implications. *The Laryngoscope* **120**, 1756-1772
140. Koh, J., Cho, N., Kong, G., Lee, J., and Yoon, K. (1998) p53 mutations and human papillomavirus DNA in oral squamous cell carcinoma: correlation with apoptosis. *British journal of cancer* **78**, 354
141. Laco, J., Vosmikova, H., Novakova, V., Celakovsky, P., Dolezalova, H., Tucek, L., Nekvindova, J., Vosmik, M., Cermakova, E., and Ryska, A. (2011) The role of high-risk human papillomavirus infection in oral and oropharyngeal squamous cell carcinoma in non-smoking and non-drinking patients: a clinicopathological and molecular study of 46 cases. *Virchows Archiv* **458**, 179-187
142. Syrjänen, S. (2010) The role of human papillomavirus infection in head and neck cancers. *Annals of oncology* **21**, vii243-vii245
143. Michaud, D. S., Langevin, S. M., Eliot, M., Nelson, H. H., Pawlita, M., McClean, M. D., and Kelsey, K. T. (2014) High-risk HPV types and head and neck cancer. *International Journal of Cancer* **135**, 1653-1661
144. Melchers, L., Mastik, M., Cameron, B. S., van Dijk, B., de Bock, G., van der Laan, B., van der Vegt, B., Speel, E., Roodenburg, J., and Witjes, M. (2015) Detection of HPV-associated oropharyngeal tumours in a 16-year cohort: more than meets the eye. *British journal of cancer* **112**, 1349-1357
145. Khanal, S., Joh, J., Kwon, A. M., Zahin, M., Perez, C. A., Dunlap, N. E., Silverman, C. L., Tennant, P. A., Potts, K. L., Kloecker, G. H., Bumpous, J. M., Ghim, S. J., Jenson, A. B., and Redman, R. A. (2015) Human papillomavirus E7 serology and association with p16 immunohistochemistry in squamous cell carcinoma of the head and neck. *Experimental and molecular pathology* **99**, 335-340
146. Kirnbauer, R., Hubbert, N. L., Wheeler, C. M., Becker, T. M., Lowy, D. R., and Schiller, J. T. (1994) A virus-like particle enzyme-linked immunosorbent assay detects serum antibodies in a majority of women infected with human papillomavirus type 16. *Journal of the National Cancer Institute* **86**, 494-499
147. Ferguson, M., Heath, A., Johnes, S., Pagliusi, S., and Dillner, J. (2006) Results of the first WHO international collaborative study on the standardization of the detection of antibodies to human papillomaviruses. *International journal of cancer* **118**, 1508-1514

148. Coseo, S. E., Porras, C., Dodd, L. E., Hildesheim, A., Rodriguez, A. C., Schiffman, M., Herrero, R., Wacholder, S., Gonzalez, P., and Sherman, M. E. (2011) Evaluation of the polyclonal ELISA HPV serology assay as a biomarker for HPV exposure. *Sexually transmitted diseases* **38**, 976
149. Smith, E. M., Ritchie, J. M., Pawlita, M., Rubenstein, L. M., Haugen, T. H., Turek, L. P., and Hamsikova, E. (2007) Human papillomavirus seropositivity and risks of head and neck cancer. *International journal of cancer* **120**, 825-832
150. Herrero, R., Castellsagué, X., Pawlita, M., Lissowska, J., Kee, F., Balaram, P., Rajkumar, T., Sridhar, H., Rose, B., and Pintos, J. (2003) Human papillomavirus and oral cancer: the International Agency for Research on Cancer multicenter study. *Journal of the National Cancer Institute* **95**, 1772-1783
151. Schwartz, S. M., Daling, J. R., Madeleine, M. M., Doody, D. R., Fitzgibbons, E. D., Wipf, G. C., Carter, J. J., Mao, E.-J., Huang, S., and Beckmann, A. M. (1998) Oral cancer risk in relation to sexual history and evidence of human papillomavirus infection. *Journal of the National Cancer Institute* **90**, 1626-1636
152. Anderson, K. S., Wong, J., D'Souza, G., Riemer, A. B., Lorch, J., Haddad, R., Pai, S., Longtine, J., McClean, M., and LaBaer, J. (2011) Serum antibodies to the HPV16 proteome as biomarkers for head and neck cancer. *British journal of cancer* **104**, 1896-1905
153. Zumbach, K., Hoffmann, M., Kahn, T., Bosch, F., Gottschlich, S., Görögh, T., Rudert, H., and Pawlita, M. (2000) Antibodies against oncoproteins E6 and E7 of human papillomavirus types 16 and 18 in patients with head-and-neck squamous-cell carcinoma. *International journal of cancer* **85**, 815-818
154. Furniss, C., McClean, M., Smith, J., Bryan, J., Applebaum, K., Nelson, H., Posner, M., and Kelsey, K. (2009) Human papillomavirus 6 seropositivity is associated with risk of head and neck squamous cell carcinoma, independent of tobacco and alcohol use. *Annals of oncology* **20**, 534-541
155. Smith, E. M., Rubenstein, L. M., Ritchie, J. M., Lee, J. H., Haugen, T. H., Hamsikova, E., and Turek, L. P. (2008) Does pretreatment seropositivity to human papillomavirus have prognostic significance for head and neck cancers? *Cancer Epidemiology Biomarkers & Prevention* **17**, 2087-2096
156. Koslabova, E., Hamsikova, E., Salakova, M., Klozar, J., Foltynova, E., Salkova, E., Rotnaglova, E., Ludvikova, V., and Tachezy, R. (2013) Markers of HPV infection and survival in patients with head and neck tumors. *International journal of cancer* **133**, 1832-1839
157. Rubenstein, L. M., Smith, E. M., Pawlita, M., Haugen, T. H., Hamsikova, E., and Turek, L. P. (2011) Human papillomavirus serologic follow-up response and relationship to survival in head and neck cancer: a case-comparison study. *Infectious agents and cancer* **6**, 9
158. Storey, R., Joh, J., Kwon, A., Jenson, A. B., Ghim, S.-j., and Kloecker, G. H. (2013) Detection of immunoglobulin G against E7 of human papillomavirus in non-small-cell lung cancer. *Journal of oncology* **2013**
159. Baay, M., Duk, J., Burger, M., De Bruijn, H., Stolz, E., and Herbrink, P. (1999) Humoral immune response against proteins E6 and E7 in cervical carcinoma patients positive for human papilloma virus type 16 during treatment and follow-up. *European Journal of Clinical Microbiology and Infectious Diseases* **18**, 126-132
160. Di Lonardo, A., Marcante, M. L., Poggiali, F., and Venuti, A. (1998) HPV 16 E7 antibody levels in cervical cancer patients: before and after treatment. *Journal of medical virology* **54**, 192-195
161. Hamšíková, E., Ludvíková, V., Tachezy, R., Kovařík, J., Břoušková, L., and Vonka, V. (2000) Longitudinal follow-up of antibody response to selected antigens of human

papillomaviruses and herpesviruses in patients with invasive cervical carcinoma. *International journal of cancer* **86**, 351-355
162. Ghim, S., Basu, P. S., and Jenson, A. (2002) Cervical cancer: etiology, pathogenesis, treatment, and future vaccines. *Asian Pacific journal of cancer prevention : APJCP* **3**, 207-214
163. Liang, C., Marsit, C. J., McClean, M. D., Nelson, H. H., Christensen, B. C., Haddad, R. I., Clark, J. R., Wein, R. O., Grillone, G. A., and Houseman, E. A. (2012) Biomarkers of HPV in head and neck squamous cell carcinoma. *Cancer research* **72**, 5004-5013
164. D'Souza, G., Kreimer, A. R., Viscidi, R., Pawlita, M., Fakhry, C., Koch, W. M., Westra, W. H., and Gillison, M. L. (2007) Case–control study of human papillomavirus and oropharyngeal cancer. *New England Journal of Medicine* **356**, 1944-1956
165. Peitsaro, P., Johansson, B., and Syrjänen, S. (2002) Integrated human papillomavirus type 16 is frequently found in cervical cancer precursors as demonstrated by a novel quantitative real-time PCR technique. *Journal of clinical microbiology* **40**, 886-891
166. Schmittgen, T. D., and Livak, K. J. (2008) Analyzing real-time PCR data by the comparative CT method. *Nature protocols* **3**, 1101-1108
167. Livak, K., and Schmittgen, T. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT Method. Methods.[Internet]. 25: 402–408.
168. Akagi, K., Li, J., Broutian, T. R., Padilla-Nash, H., Xiao, W., Jiang, B., Rocco, J. W., Teknos, T. N., Kumar, B., and Wangsa, D. (2014) Genome-wide analysis of HPV integration in human cancers reveals recurrent, focal genomic instability. *Genome research* **24**, 185-199
169. Ramamoorthy, S., Liu, Y.-T., Luo, L., Miyai, K., Lu, Q., and Carethers, J. M. (2010) Detection of multiple human papillomavirus genotypes in anal carcinoma. *Infectious agents and cancer* **5**, 17
170. Tang, A. L., Hauff, S. J., Owen, J. H., Graham, M. P., Czerwinski, M. J., Park, J. J., Walline, H., Papagerakis, S., Stoerker, J., McHugh, J. B., Chepeha, D. B., Bradford, C. R., Carey, T. E., and Prince, M. E. (2012) UM-SCC-104: a new human papillomavirus-16-positive cancer stem cell-containing head and neck squamous cell carcinoma cell line. *Head & neck* **34**, 1480-1491
171. Brenner, J. C., Graham, M. P., Kumar, B., Saunders, L. M., Kupfer, R., Lyons, R. H., Bradford, C. R., and Carey, T. E. (2010) Genotyping of 73 UM-SCC head and neck squamous cell carcinoma cell lines. *Head & neck* **32**, 417-426
172. Young, R. J., Rischin, D., Fisher, R., McArthur, G. A., Fox, S. B., Peters, L. J., Corry, J., Lim, A., Waldeck, K., and Solomon, B. (2011) Relationship between epidermal growth factor receptor status, p16INK4A, and outcome in head and neck squamous cell carcinoma. *Cancer Epidemiology Biomarkers & Prevention* **20**, 1230-1237
173. Kang, H., Kiess, A., and Chung, C. H. (2015) Emerging biomarkers in head and neck cancer in the era of genomics. *Nature reviews. Clinical oncology* **12**, 11-26
174. Zhang, C., Deng, Z., Pan, X., Uehara, T., Suzuki, M., and Xie, M. (2015) Effects of Methylation Status of CpG Sites within the HPV16 Long Control Region on HPV16-Positive Head and Neck Cancer Cells. *PloS one* **10**, e0141245
175. Olthof, N. C., Huebbers, C. U., Kolligs, J., Henfling, M., Ramaekers, F. C., Cornet, I., van Lent-Albrechts, J. A., Stegmann, A. P., Silling, S., Wieland, U., Carey, T. E., Walline, H. M., Gollin, S. M., Hoffmann, T. K., de Winter, J., Kremer, B., Klussmann, J. P., and Speel, E. J. (2015) Viral load, gene expression and mapping of viral integration sites in HPV16-associated HNSCC cell lines. *Int J Cancer* **136**, E207-218
176. Cheung, J. L., Cheung, T. H., Yu, M. Y., and Chan, P. K. (2013) Virological characteristics of cervical cancers carrying pure episomal form of HPV16 genome. *Gynecol Oncol* **131**, 374-379

177. Mazumder Indra, D., Singh, R. K., Mitra, S., Dutta, S., Chakraborty, C., Basu, P. S., Mondal, R. K., Roychoudhury, S., and Panda, C. K. (2011) Genetic and epigenetic changes of HPV16 in cervical cancer differentially regulate E6/E7 expression and associate with disease progression. *Gynecol Oncol* **123**, 597-604
178. Chaiwongkot, A., Vinokurova, S., Pientong, C., Ekalaksananan, T., Kongyingyoes, B., Kleebkaow, P., Chumworathayi, B., Patarapadungkit, N., Reuschenbach, M., and von Knebel Doeberitz, M. (2013) Differential methylation of E2 binding sites in episomal and integrated HPV 16 genomes in preinvasive and invasive cervical lesions. *International Journal of Cancer* **132**, 2087-2094
179. Ghosh, D. D., Bhattacharjee, B., Sen, S., Premi, L., Mukhopadhyay, I., Chowdhury, R. R., Roy, S., and Sengupta, S. (2012) Some novel insights on HPV16 related cervical cancer pathogenesis based on analyses of LCR methylation, viral load, E7 and E2/E4 expressions. *PloS one* **7**, e44678
180. Maiti, G. P., Mondal, P., Mukherjee, N., Ghosh, A., Ghosh, S., Dey, S., Chakrabarty, J., Roy, A., Biswas, J., and Roychoudhury, S. (2013) Overexpression of EGFR in head and neck squamous cell carcinoma is associated with inactivation of SH3GL2 and CDC25A genes. *PloS one* **8**, e63440
181. Lassen, P., Overgaard, J., and Eriksen, J. G. (2013) Expression of EGFR and HPV-associated p16 in oropharyngeal carcinoma: correlation and influence on prognosis after radiotherapy in the randomized DAHANCA 5 and 7 trials. *Radiotherapy and Oncology* **108**, 489-494
182. Zimmermann, M., Zouhair, A., Azria, D., and Ozsahin, M. (2006) The epidermal growth factor receptor (EGFR) in head and neck cancer: its role and treatment implications. *Radiation oncology* **1**, 1
183. Cullen, A. P., Reid, R., Campion, M., and Lörincz, A. (1991) Analysis of the physical state of different human papillomavirus DNAs in intraepithelial and invasive cervical neoplasm. *Journal of virology* **65**, 606-612
184. Daniel, B., Mukherjee, G., Seshadri, L., Vallikad, E., and Krishna, S. (1995) Changes in the physical state and expression of human papillomavirus type 16 in the progression of cervical intraepithelial neoplasia lesions analysed by PCR. *Journal of general virology* **76**, 2589-2593
185. Klaes, R., Woerner, S. M., Ridder, R., Wentzensen, N., Duerst, M., Schneider, A., Lotz, B., Melsheimer, P., and von Knebel Doeberitz, M. (1999) Detection of high-risk cervical intraepithelial neoplasia and cervical cancer by amplification of transcripts derived from integrated papillomavirus oncogenes. *Cancer research* **59**, 6132-6136
186. Pirami, L., Giache, V., and Becciolini, A. (1997) Analysis of HPV16, 18, 31, and 35 DNA in pre-invasive and invasive lesions of the uterine cervix. *Journal of clinical pathology* **50**, 600-604
187. Handy, D. E., Castro, R., and Loscalzo, J. (2011) Epigenetic modifications basic mechanisms and role in cardiovascular disease. *Circulation* **123**, 2145-2156
188. Herman, J. G., and Baylin, S. B. (2003) Gene silencing in cancer in association with promoter hypermethylation. *New England Journal of Medicine* **349**, 2042-2054
189. Holliday, R. (2005) DNA methylation and epigenotypes. *Biochemistry (Moscow)* **70**, 500-504
190. Sen, G. L., Reuter, J. A., Webster, D. E., Zhu, L., and Khavari, P. A. (2010) DNMT1 maintains progenitor function in self-renewing somatic tissue. *Nature* **463**, 563-567
191. Leonard, S., Collins, S., Pereira, M., Diyaf, A., Constandinou-Williams, C., Young, L., Roberts, S., and Woodman, C. (2012) Oncogenic human papillomavirus imposes an instructive pattern of DNA methylation changes which parallel the natural history of cervical HPV infection in young women. *Carcinogenesis*, bgs157

192. Wentzensen, N., Sherman, M. E., Schiffman, M., and Wang, S. S. (2009) Utility of methylation markers in cervical cancer early detection: appraisal of the state-of-the-science. *Gynecologic oncology* **112**, 293-299
193. Demokan, S., and Dalay, N. (2011) Role of DNA methylation in head and neck cancer. *Clinical epigenetics* **2**, 123-150
194. Koutsimpelas, D., Pongsapich, W., Heinrich, U., Mann, S., Mann, W. J., and Brieger, J. (2012) Promoter methylation of MGMT, MLH1 and RASSF1A tumor suppressor genes in head and neck squamous cell carcinoma: pharmacological genome demethylation reduces proliferation of head and neck squamous carcinoma cells. *Oncology reports* **27**, 1135-1141
195. Sartor, M. A., Dolinoy, D. C., Jones, T. R., Colacino, J. A., Prince, M. E., Carey, T. E., and Rozek, L. S. (2011) Genome-wide methylation and expression differences in HPV (+) and HPV (-) squamous cell carcinoma cell lines are consistent with divergent mechanisms of carcinogenesis. *Epigenetics* **6**, 777-787
196. Nikolaidis, C., Nena, E., Panagopoulou, M., Balgkouranidou, I., Karaglani, M., Chatzaki, E., Agorastos, T., and Constantinidis, T. C. (2015) PAX1 methylation as an auxiliary biomarker for cervical cancer screening: A meta-analysis. *Cancer epidemiology* **39**, 682-686
197. Marsit, C. J., McClean, M. D., Furniss, C. S., and Kelsey, K. T. (2006) Epigenetic inactivation of the SFRP genes is associated with drinking, smoking and HPV in head and neck squamous cell carcinoma. *International journal of cancer* **119**, 1761-1766
198. Tokumaru, Y., Yamashita, K., Osada, M., Nomoto, S., Sun, D.-I., Xiao, Y., Hoque, M. O., Westra, W. H., Califano, J. A., and Sidransky, D. (2004) Inverse correlation between cyclin A1 hypermethylation and p53 mutation in head and neck cancer identified by reversal of epigenetic silencing. *Cancer research* **64**, 5982-5987
199. Bertonha, F. B., de Camargo Barros Filho, M., Kuasne, H., dos Reis, P. P., da Costa Prando, E., Muñoz, J. J. A. M., Roffé, M., Hajj, G. N. M., Kowalski, L. P., and Rainho, C. A. (2015) PHF21B as a candidate tumor suppressor gene in head and neck squamous cell carcinomas. *Molecular oncology* **9**, 450-462
200. Tsunematsu, T., Kudo, Y., Iizuka, S., Ogawa, I., Fujita, T., Kurihara, H., Abiko, Y., and Takata, T. (2009) RUNX3 has an oncogenic role in head and neck cancer. *PloS one* **4**, e5892
201. Dong, S. M., Sun, D.-I., Benoit, N. E., Kuzmin, I., Lerman, M. I., and Sidransky, D. (2003) Epigenetic inactivation of RASSF1A in head and neck cancer. *Clinical cancer research* **9**, 3635-3640
202. Choudhury, J. H., and Ghosh, S. K. (2015) Promoter hypermethylation profiling identifies subtypes of head and neck cancer with distinct viral, environmental, genetic and survival characteristics. *PloS one* **10**, e0129808
203. Chen, K. M., Stephen, J. K., Havard, S., Mahan, M., Divine, G., and Worsham, M. J. (2015) IGSF4 Methylation as an Independent Marker of Human Papillomavirus–Positive Oropharyngeal Squamous Cell Carcinoma. *JAMA Otolaryngology–Head & Neck Surgery* **141**, 257-263
204. Agodi, A., Barchitta, M., Quattrocchi, A., Maugeri, A., and Vinciguerra, M. (2015) DAPK1 Promoter Methylation and Cervical Cancer Risk: A Systematic Review and a Meta-Analysis. *PloS one* **10**, e0135078
205. Henken, F. E., Wilting, S. M., Overmeer, R. M., van Rietschoten, J. G., Nygren, A. O., Errami, A., Schouten, J. P., Meijer, C. J., Snijders, P. J., and Steenbergen, R. D. (2007) Sequential gene promoter methylation during HPV-induced cervical carcinogenesis. *British journal of cancer* **97**, 1457-1464

206. Lee, J., Wysocki, P. T., Topaloglu, O., Maldonado, L., Brait, M., Begum, S., Moon, D., Kim, M. S., Califano, J. A., and Sidransky, D. (2015) Epigenetic silencing of S100A2 in bladder and head and neck cancers. *Oncoscience* **2**, 410
207. Colacino, J. A., Dolinoy, D. C., Duffy, S. A., Sartor, M. A., Chepeha, D. B., Bradford, C. R., McHugh, J. B., Patel, D. A., Virani, S., and Walline, H. M. (2013) Comprehensive analysis of DNA methylation in head and neck squamous cell carcinoma indicates differences by survival and clinicopathologic characteristics. *PloS one* **8**, e54742
208. Koffler, J., Sharma, S., and Hess, J. (2014) Predictive value of epigenetic alterations in head and neck squamous cell carcinoma. *Molecular & Cellular Oncology* **1**, e954827
209. Peng, J., Chen, B., Shen, Z., Deng, H., Liu, D., Xie, X., Gan, X., Xu, X., Huang, Z., and Chen, J. (2015) DNA promoter hypermethylation contributes to down-regulation of galactocerebrosidase gene in lung and head and neck cancers. *International journal of clinical and experimental pathology* **8**, 11042
210. Stephen, J. K., Chen, K. M., Havard, S., Harris, G., and Worsham, M. J. (2012) Promoter methylation in head and neck tumorigenesis. *Cancer Epigenetics: Methods and Protocols*, 187-206
211. Weiss, D., Basel, T., Sachse, F., Braeuninger, A., and Rudack, C. (2011) Promoter methylation of cyclin A1 is associated with human papillomavirus 16 induced head and neck squamous cell carcinoma independently of p53 mutation. *Molecular carcinogenesis* **50**, 680-688
212. van Kempen, P. M., Noorlag, R., Braunius, W. W., Stegeman, I., Willems, S. M., and Grolman, W. (2014) Differences in methylation profiles between HPV-positive and HPV-negative oropharynx squamous cell carcinoma: a systematic review. *Epigenetics* **9**, 194-203
213. Lechner, M., Fenton, T., West, J., Wilson, G., Feber, A., Henderson, S., Thirlwell, C., Dibra, H. K., Jay, A., and Butcher, L. (2013) Identification and functional validation of HPV-mediated hypermethylation in head and neck squamous cell carcinoma. *Genome med* **5**, 15
214. Gubanova, E., Brown, B., Ivanov, S. V., Helleday, T., Mills, G. B., Yarbrough, W. G., and Issaeva, N. (2012) Downregulation of SMG-1 in HPV-positive head and neck squamous cell carcinoma due to promoter hypermethylation correlates with improved survival. *Clinical Cancer Research* **18**, 1257-1267
215. Kempen, P. M., Bockel, L., Braunius, W. W., Moelans, C. B., Olst, M., Jong, R., Stegeman, I., Diest, P. J., Grolman, W., and Willems, S. M. (2014) HPV-positive oropharyngeal squamous cell carcinoma is associated with TIMP3 and CADM1 promoter hypermethylation. *Cancer medicine* **3**, 1185-1196
216. Li, L. C., and Dahiya, R. (2002) MethPrimer: designing primers for methylation PCRs. *Bioinformatics* **18**, 1427-1431
217. Stresemann, C., and Lyko, F. (2008) Modes of action of the DNA methyltransferase inhibitors azacytidine and decitabine. *Int J Cancer* **123**, 8-13
218. Rønneberg, J. A., Fleischer, T., Solvang, H. K., Nordgard, S. H., Edvardsen, H., Potapenko, I., Nebdal, D., Daviaud, C., Gut, I., and Bukholm, I. (2011) Methylation profiling with a panel of cancer related genes: association with estrogen receptor, TP53 mutation status and expression subtypes in sporadic breast cancer. *Molecular oncology* **5**, 61-76
219. Toyoda, H., Komurasaki, T., Uchida, D., Takayama, Y., Isobe, T., Okuyama, T., and Hanada, K. (1995) Epiregulin. A novel epidermal growth factor with mitogenic activity for rat primary hepatocytes. *The Journal of biological chemistry* **270**, 7495-7500

220. Komurasaki, T., Toyoda, H., Uchida, D., and Morimoto, S. (1997) Epiregulin binds to epidermal growth factor receptor and ErbB-4 and induces tryosine phosphorylation of epidermal growth factor receptor, ErbB-2, ErbB-3 and ErbB-4. *Oncogene* **15**
221. Lee, D., Pearsall, R. S., Das, S., Dey, S. K., Godfrey, V. L., and Threadgill, D. W. (2004) Epiregulin is not essential for development of intestinal tumors but is required for protection from intestinal damage. *Molecular and cellular biology* **24**, 8907-8916
222. TOYODA, H., KOMURASAKI, T., UCHIDA, D., and MORIMOTO, S. (1997) Distribution of mRNA for human epiregulin, a differentially expressed member of the epidermal growth factor family. *Biochemical Journal* **326**, 69-75
223. Shirakata, Y., Komurasaki, T., Toyoda, H., Hanakawa, Y., Yamasaki, K., Tokumaru, S., Sayama, K., and Hashimoto, K. (2000) Epiregulin, a novel member of the epidermal growth factor family, is an autocrine growth factor in normal human keratinocytes. *Journal of Biological Chemistry* **275**, 5748-5753
224. Taylor, D. S., Cheng, X., Pawlowski, J. E., Wallace, A. R., Ferrer, P., and Molloy, C. J. (1999) Epiregulin is a potent vascular smooth muscle cell-derived mitogen induced by angiotensin II, endothelin-1, and thrombin. *Proceedings of the National Academy of Sciences* **96**, 1633-1638
225. Toyoda, H., Komurasaki, T., Ikeda, Y., Yoshimoto, M., and Morimoto, S. (1995) Molecular cloning of mouse epiregulin, a novel epidermal growth factor-related protein, expressed in the early stage of development. *FEBS letters* **377**, 403-407
226. Hu, K., Li, S.-l., Gan, Y.-h., Wang, C.-y., and Yu, G.-y. (2009) Epiregulin promotes migration and invasion of salivary adenoid cystic carcinoma cell line SACC-83 through activation of ERK and Akt. *Oral oncology* **45**, 156-163
227. Zhuang, S., Yan, Y., Daubert, R. A., and Schnellmann, R. G. (2007) Epiregulin promotes proliferation and migration of renal proximal tubular cells. *American Journal of Physiology-Renal Physiology* **293**, F219-F226
228. Riese, D. J., 2nd, and Cullum, R. L. (2014) Epiregulin: roles in normal physiology and cancer. *Seminars in cell & developmental biology* **28**, 49-56
229. Eltarhouny, S., Elsawy, W., Radpour, R., Hahn, S., Holzgreve, W., and Zhong, X. (2008) Genes controlling spread of breast cancer to lung “gang of 4”. *Experimental oncology* **30**, 91-95
230. Yun, J., Song, S. H., Park, J., Kim, H. P., Yoon, Y. K., Lee, K. H., Han, S. W., Oh, D. Y., Im, S. A., Bang, Y. J., and Kim, T. Y. (2012) Gene silencing of EREG mediated by DNA methylation and histone modification in human gastric cancers. *Laboratory investigation; a journal of technical methods and pathology* **92**, 1033-1044
231. Shigeishi, H., Higashikawa, K., Hiraoka, M., Fujimoto, S., Mitani, Y., Ohta, K., Takechi, M., and Kamata, N. (2008) Expression of epiregulin, a novel epidermal growth factor ligand associated with prognosis in human oral squamous cell carcinomas. *Oncology reports* **19**, 1557-1564
232. John, K., Lahoti, T. S., Wagner, K., Hughes, J. M., and Perdew, G. H. (2014) The Ah receptor regulates growth factor expression in head and neck squamous cell carcinoma cell lines. *Molecular carcinogenesis* **53**, 765-776
233. Dok, R., and Nuyts, S. (2016) HPV Positive Head and Neck Cancers: Molecular Pathogenesis and Evolving Treatment Strategies. *Cancers* **8**, 41
234. Jonker, D., Karapetis, C., Harbison, C., O'Callaghan, C., Tu, D., Simes, R., Malone, D., Langer, C., Tebbutt, N., and Price, T. (2014) Epiregulin gene expression as a biomarker of benefit from cetuximab in the treatment of advanced colorectal cancer. *British journal of cancer* **110**, 648-655
235. Oshima, G., Wennerberg, J., Yamatodani, T., Kjellen, E., Mineta, H., Johnsson, A., and Ekblad, L. (2012) Autocrine epidermal growth factor receptor ligand production and

cetuximab response in head and neck squamous cell carcinoma cell lines. *Journal of cancer research and clinical oncology* **138**, 491-499
236. Badar, T., Kantarjian, H. M., Ravandi, F., Jabbour, E., Borthakur, G., Cortes, J. E., Pemmaraju, N., Pierce, S. R., Newberry, K. J., Daver, N., and Verstovsek, S. (2015) Therapeutic benefit of decitabine, a hypomethylating agent, in patients with high-risk primary myelofibrosis and myeloproliferative neoplasm in accelerated or blastic/acute myeloid leukemia phase. *Leukemia research* **39**, 950-956
237. Daskalakis, M., Blagitko-Dorfs, N., and Hackanson, B. (2010) Decitabine. in *Small Molecules in Oncology*, Springer. pp 131-157
238. Viet, C. T., Dang, D., Ye, Y., Ono, K., Campbell, R. R., and Schmidt, B. L. (2014) Demethylating drugs as novel analgesics for cancer pain. *Clinical Cancer Research* **20**, 4882-4893
239. Kantarjian, H., Issa, J. P. J., Rosenfeld, C. S., Bennett, J. M., Albitar, M., DiPersio, J., Klimek, V., Slack, J., De Castro, C., and Ravandi, F. (2006) Decitabine improves patient outcomes in myelodysplastic syndromes. *Cancer* **106**, 1794-1803
240. Viet, C. T., Dang, D., Achdjian, S., Ye, Y., Katz, S. G., and Schmidt, B. L. (2014) Decitabine rescues cisplatin resistance in head and neck squamous cell carcinoma. *PloS one* **9**, e112880
241. Primeau, M., Gagnon, J., and Momparler, R. L. (2003) Synergistic antineoplastic action of DNA methylation inhibitor 5-AZA-2'-deoxycytidine and histone deacetylase inhibitor depsipeptide on human breast carcinoma cells. *Int J Cancer* **103**, 177-184
242. Murakami, J., Asaumi, J., Maki, Y., Tsujigiwa, H., Kuroda, M., Nagai, N., Yanagi, Y., Inoue, T., Kawasaki, S., Tanaka, N., Matsubara, N., and Kishi, K. (2004) Effects of demethylating agent 5-aza-2(')-deoxycytidine and histone deacetylase inhibitor FR901228 on maspin gene expression in oral cancer cell lines. *Oral oncology* **40**, 597-603
243. Shukla, P., Solanki, A., Ghosh, K., and Vundinti, B. R. (2013) DNA interstrand cross-link repair: understanding role of Fanconi anemia pathway and therapeutic implications. *European journal of haematology* **91**, 381-393
244. Rieckmann, T., Tribius, S., Grob, T. J., Meyer, F., Busch, C.-J., Petersen, C., Dikomey, E., and Kriegs, M. (2013) HNSCC cell lines positive for HPV and p16 possess higher cellular radiosensitivity due to an impaired DSB repair capacity. *Radiotherapy and Oncology* **107**, 242-246
245. Lu, S.-L. (2011) Fanconi Anemia/Brca Pathway and Head and Neck Squamous Cell Carcinomas.

APPENDICES

Introduction

During the course of my dissertation work, some supporting experiments were not completed and some of the studies did not produce significant results. Therefore, I had to change research aims and/or experimental focus. These results are not included in dissertation chapters and therefore are shown in the appendix.

I. Determine the presence of Fanconi anemia genes mutation(s) in HPV-positive head and neck cancer specimens

Introduction

My original thesis proposal was to study whether a defect in Fanconi Anemia (FA) - DNA repair system is associated with HPV-induced HNCs and whether this defect is linked with improved prognosis in HPV-positive HNCs. This was based on the studies which have shown a defect in FA-DNA repair pathway is implicated in hypersensitivity to DNA cross-linking agents (243) and HPV-induced HNC exhibit improved sensitivity to chemo/radio therapies (244). While there is evidence for a DNA repair defect in HNCs, it is not well documented whether this is specific to the HPV-positive environment. Sixteen proteins encoded from 16 Fanconi anemia (*FANC*) genes are involved in a DNA-repair pathway known as the Fanconi Anemia-DNA repair pathway (245) (**Figure S1**). Based on these findings, I hypothesized that HPV-positive HNCs contain mutations in Fanconi genes that are not present in HPV-negative HNCs and this defect in Fanconi anemia-DNA repair pathway is implicated in improved prognosis in HPV-positive HNCs.

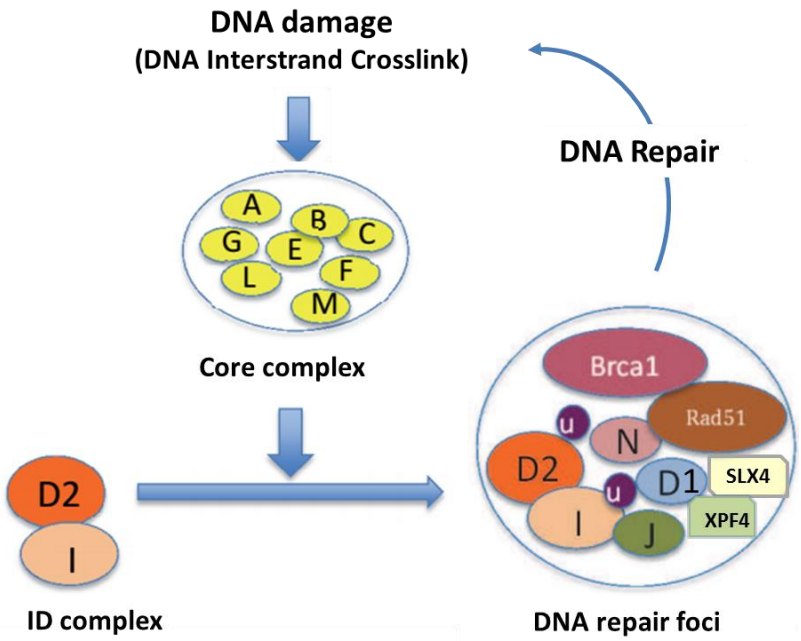

Figure S1: Fanconi anemia (FA)-DNA repair pathway showing into three groups of FA proteins: **1)** Core complex proteins (FANCA, B, C, E, F, G, L and M); which in response to DNA damage is required for mono-ubiquitination of **2)** ID complex (FANCI and FANCD2); and **3)** DNA repair foci proteins [FANCD1/BRCA2, FANCJ/BRIP1, FANCN/PALB2, FANCO/ RAD51C, FANCP/SLX4 and FANCQ/XPF4/ERCC4] participate directly in DNA interstrand crosslink repair. Accessory proteins such as MHF1/2 and FAAP24 (not shown here) help FANCM to recruit core complex to the sites of DNA damage. The monoubiquitinated FANCD2 is the center of the pathway and a measure of functional FA pathway. BRCA1 is currently not considered to be a true FA protein though it is an essential part of the FA-DNA repair pathway.

Experimental Procedures

I had a plan to test all coding exons of 16 *FANC* genes (total 303 exons) by PCR-based amplification using primers spanning intron-exon junctions. I had started with the amplification and sequencing of 9 exons of *FANCO/RAD51C*; *14 exons of FANCL*; *23 exons of FANCM*; *14 exons of FANCG* and *43 exons of FANCD2.* Primers, PCR conditions and the target PCR product for each exon of *FANC* genes were tested first by using HeLa cell DNA. By optimizing PCR conditions, I amplified the respective exons in the HNC samples. Then, target PCR products were excised from an agarose gel, purified, and sequenced.

When a gene is known to produce multiple transcripts, the primers for shared exons and primers unique for the spliced exon were designed. Multiple primer pairs were employed if the coding exons were larger than 450bp. Primers were designed using Primer 3 software or IDT PrimerQuest tool to cover at least 15bp at each 5'and 3' sides of the exons. I ordered primers (either forward or reverse) tagged with M13 primer (5'-GTAAAACGACGGCCAGT-3') so that all amplified products can be sequenced using a single M13 primer.

Data analysis

Sequencing data was analyzed for variant detection using Mutation Surveyor software (SoftGenetics LLC., PA, USA). All sequences were compared to the related NCBI reference sequences. The chromatograms of all the computationally determined variants were checked manually for confirming the existence of those variants. SeqMan Pro program (Lasergene 12, DNAstar Inc., Wisconsin) was used to align and compare the sequences from all the samples analyzed. The identified variants were searched in NCBI dbSNP for Human to find their related rs#, and those, which was not found, was considered as novel variants. Human Splicing Finder website (http://www.umd.be/HSF/) was used to determine whether the detected intronic and exonic mutations lead to splicing defects.

All insertion and deletions in the gene coding region, nonsense variants, and variants located at the splice site consensus sequences were considered as potentially deleterious. Missense variants first were searched in UniProt Database for determining their functional effects. If their functional effect was not known, bioinformatics tools were utilized to predict their effect. For predicting the functional effect of the missense variants, PolyPhen and SIFT tools were used. Missense variants predicted to be deleterious by Polyphen or SIFT algorithms and having a minimum allele frequency (MAF) >0.5% was considered as potentially deleterious variants. The MAF > 0.5% was used to exclude singleton mutations (private mutations seen in a single individual) which do not provide enough power for analysis. The predicted potentially deleterious variants were planned to genotype in more cases and controls.

Following mutational analysis, each putative mutation was independently re-amplified in tumor DNA (to eliminate artifacts). I compared the sequencing results of each patient's tumor

to their matched tissue samples in order to eliminate background germline variations and to focus on somatic alterations unique to the tumor genome. The comparison was done with HPV-negative samples to establish a link between *FANC* mutation and HPV infection.

Results and Discussion

I analyzed two HPV-positive HNC samples with their matched normal tissues, and two HPV− samples (altogether 6 samples). In 6 samples, I had sequenced 9 exons of *FANCO/RAD51C*; *14 exons of FANCL*; *23 exons of FANCM*; *14 exons of FANCG* and *43 exons of FANCD2*. I was unable to get useful sequencing results from *FANCD2* Exons 18, 19, 20, 22 and 26 amplified products. Although the primers were designed to target exons, some intergenic and intron regions adjacent to exons were also amplified and sequenced. Variants unique to HPV-positive HNC cases are shown in **Table S1** and **Table S2**.

The majority of mutations that were observed were heterozygous synonymous. Mutations that were heterozygous non-synonymous were mostly present in both tumor and normal matched tissues, showing germline variations instead of somatic alterations unique to the tumor genome. These mutations include *FANCD2* c.2219C>CT (or p.P714PL); *RAD51C* c.439C>CA (or p.Q133QK); *FANCM* ----c.437A>AC (or p.T118TP) c.5268C>CG (or p.A1728AG) and c.5273C>CG (or p.Q1730QE); *FANCG* --- c.816G>GC (or p.E108ED), c.923G>GC (or p.G144GA) and c.2197G>GC (or p.A569AP).

Heterozygous non-synonymous mutation of *FANCG* c.819G>GC (or p.Q109QH) and heterozygous deletion of *FANCM* c.2379het_delT were found in HPV+ tumors but not in matched normal. However, these mutations were not observed in additional HPV-positive cases and *FANCG* c.819G>GC was also present in additional HPV-negative cases; suggesting these alterations were not exclusively specific for the HPV-positive environment.

Most of the intronic variants were heterozygous and were also present in matched normal. Heterozygous deletion of RAD51C c.947-74het_delG was found in one HPV-positive tumor but there wasn't predicted potential splice site, making this mutation non-significant.

Table S1: Exonic variants identified in 5 *FANC* genes in DNA of only HPV-positive HNC cases

No.	Gene	Exon	Nucleotide Change*	Amino Acid Change	Mutation type	Total Samples
1	*FANCD2*	14	c.1200A>AG	p.V374VV	Heterozygous synonymous	2 (tumor+ matched normal)
2	*FANCD2*	17	c.1587C>CT	p.N503NN	Heterozygous synonymous	2 (tumor+ matched normal)
3	*FANCD2*	23	c.2219C>CT	p.P714PL	Heterozygous non-synonymous	2 (tumor+ matched normal)
4	*FANCD2*	42	c.4176T>TG	p.L1366LL	Heterozygous synonymous	2 (tumor+ matched normal)
5	*RAD51C*	2	c.439C>CA	p.Q133QK	Heterozygous non-synonymous	2 (tumor+ matched normal)
6	*FANCL*	6	37214T>TG	p.G145GG	Heterozygous synonymous	4 (tumors+ matched normal)
7	*FANCM*	1	c.437A>AC,	p.T118TP	Heterozygous non-synonymous	4 (tumors+ matched normal)
8	*FANCM*	1	c.484T>TC	p.P133PP	Heterozygous synonymous	2 (tumor+ matched normal)
9	*FANCM*	13	c.2379het_delT		Heterozygous deletion	1 (tumor)
10	*FANCM*	20	c.5268C>CG	p.A1728AG	Heterozygous non-synonymous	4 (tumors+ matched normal)
11	*FANCM*	20	c.5273C>CG	p.Q1730QE	Heterozygous non-synonymous	4 (tumors+ matched normal)
12	*FANCM*	21	c.5470T>TG	p.C1795CW	Heterozygous non-synonymous	2 (tumor+ matched normal)
13	*FANCM*	21	c.5550T>TG	p.V1822VG	Heterozygous non-synonymous	4 (tumors+ matched normal)
14	*FANCM*	21	c.5554T>TG	p.G1823GG	Heterozygous synonymous	3 (2 tumor+ 1 matched normal)
15	*FANCM*	23	c.6749A>AC		Heterozygous	3 (2 tumors+ 1 matched normal)
16	*FANCM*	23	c.6880A>AC		Heterozygous	4 (tumors+ matched normal)
17	*FANCM*	23	c.6883T>TC		Heterozygous	3 (2 tumors+ 1 matched normal)
18	*FANCG*	4	c.816G>GC	p.E108ED	Heterozygous non-synonymous	2 (tumor+ matched normal)
19	*FANCG*	4	c.819G>GC	p.Q109QH	Heterozygous non-synonymous	2 tumors
20	*FANCG*	4	c.923G>GC	p.G144GA	Heterozygous non-synonymous	2 (tumor+ matched normal)
21	*FANCG*	4	c.927G>GC	p.L145LL	Heterozygous synonymous	2 (tumor+ matched normal)
22	*FANCG*	13	c.2197G>GC	p.A569AP	Heterozygous non-synonymous	2 (tumor+ matched normal)

*A mutation in the mRNA region is prefixed with a “c” and a mutation in the protein sequence is prefixed with a “p”.

Table S2: Intronic variants identified in 5 *FANC* genes in only HPV-positive HNC cases

No.	Gene	Intron adjacent to exon	Nucleotide Change*	Mutation type	Total Samples	Prediction using Human Splicing Finder
1	*FANCD2*	5	c.455+155T>TG	Heterozygous	2 (tumor+ matched normal)	Potential donor splice site
2	*FANCD2*	7	c.517-16A>AG	Heterozygous	2 (tumor+ matched normal)	None
3	*FANCD2*	13	c.1068-38C>CG	Heterozygous	2 (tumor+ matched normal)	Potential acceptor splice site
4	*FANCD2*	16	c.1357-76A>G	Homozygous	1 (tumor)	Potential donor splice site
5	*FANCD2*	17	1492-149A>AG	Heterozygous	2 (tumor+ matched normal)	None
6.	*FANCD2*	30	c.2938-4A>AC	Heterozygous	3 (2 tumors+1 matched normal)	Potential acceptor splice site
7.	*FANCD2*	42	c.4263+33T>TC	Heterozygous	2 (tumor+ matched normal)	None
8	*RAD51C*	1	c.17C>CT	Heterozygous	2 (tumor+ matched normal)	None
9	*RAD51C*	7	c.947-74het_delG	Heterozygous deletion	1 (tumor)	
10	*FANCL*	6	c.537+27T>TG	Heterozygous	2 (tumor+ matched normal)	Potential acceptor splice site
11	*FANCL*	6	c.537+97A>G	Homozygous	2 (tumor+ matched normal)	None
12	*FANCL*	9	c.758-27A>C	Homozygous	2 (tumor+ matched normal)	Potential acceptor splice site
13	*FANCM*	9	c.1666+194het_delT	Heterozygous deletion	2 (tumor+ matched normal)	Potential acceptor splice site
14	*FANCM*	12	c.2245+97G>GA	Heterozygous	4 (tumors+ matched normal)	None
15	*FANCM*	21	c.5801+36T>G	Homozygous	4 (tumors+ matched normal)	Potential donor splice site
16	*FANCG*	2	c.667+24G>GC	Heterozygous	2 (tumor+ matched normal)	Potential donor splice site

*A mutation in the mRNA region is prefixed with a “c”. A mutation that is called outside of an mRNA region is assigned as plus or minus the number of bps by which it resides away from the closest nucleotide of the mRNA

Next gene sequencing data

Next gene sequencing (NGS) using Ion AmpliSeq™ Comprehensive Cancer Gene Panel (Ion Torrent, Thermo Fisher Scientific) was done in two samples (one HPV negative HNC sample and one HPV positive HNC sample). This panel contained 7 *FANC* genes--namely *FANCA*, *FANCC*, *FANCD2*, *FANCF*, *FANCG*, *BRIP1,* and *ERCC4*.

Table S3 shows SNPs (Single nucleotide polymorphisms) which were identified in the HPV-positive sample. *FANCD2* c.1179T>C and *ERCC4* intronic mutations were found in both HPV-positive and HPV-negative samples.

Interestingly, *FANCD2* (c.1214A>G missense mutation) was seen only in an HPV-positive HNC sample, not in an HPV-negative sample. Sanger sequencing was conducted for further verification. But, this mutation in that particular location was not observed in the same sample. So, *FANCD2* (c.1214A>G missense mutation) obtained from NGS seems to be a false positive SNP. This is probably because Next Gene sequencer has problems in the regions of repeated bases in the sequence. Indeed, the location of c.1214A>G mutation was found in the region where there were repeats of A (the sequence was: aagaaataag).

Table S3: SNPs identified from an HPV-positive sample

Gene	Mutation*	Classification	Genotype	Type†	Location	Coding	Amino acid Change	Variant effect
FANCD2	c.1214A>G	Suspected Deleterious	A/G	SNV	exonic	c.1214A>G	p.Asn405Ser	missense
FANCD2	c.1179T>C	Unknown	T/C	SNV	exonic	c.1179T>C	WT	synonymous
FANCD2	c.1170C>T	Unknown	C/T	SNV	exonic	c.1170C>T	WT	synonymous
ERCC4		Unknown	A/A	SNV	intronic			

*A mutation in the mRNA region is prefixed with a “c”.
†single nucleotide variants (SNVs)

Overall results obtained from mutational analysis of Fanconi-anemia-DNA repair genes suggest that mutations in *FANC* genes may not be specific to HPV-positive head and neck cancers, though further studies on larger number of specimens are required.

II. E7 knockdown or overexpression to determine whether viral oncoprotein E7 mediates promoter methylation and transcriptional inactivation of a candidate gene *EREG*

Introduction:

The work presented in chapter V suggested a hypothesis that promoter methylation and inactivation of *EREG* is mediated by HPV presence in head and neck cancers. Since HPV16 E7 oncoprotein has been shown to upregulate DNMT1 and DNMT3a (119); I rephrased the above hypothesis as − E7 oncoprotein causes promoter methylation of *EREG*. To test this hypothesis, I attempted to perform lentiviral knockdown of E7 in HPV-positive UMSCC-47 cell line and lentiviral-based overexpression of E7 in HPV-negative UMSCC-1 cell line.

Experimental Procedures

Cloning and production of lentiviral plasmids for overexpression of E7: The E7 coding region containing *Eco*RI and *Xho*I restriction sites was obtained from PCR-amplification of HPV16 purified plasmid (*i.e.* a plasmid containing full genome of HPV16 cloned in the pBR322 vector). Amplification was done using primers against the full length of E7 (forward primer with *Eco*RI site: aaagGAATTCatgcatggagatacacctac and a reverse primer with *Xho*I site: aaaaCTCGAGttatggtttctgagaacagatg). Amplified product was gel purified, digested with *Eco*RI and *Xho*I and then ligated with a pLUTdNB lentiviral vector which was also digested with the same restriction enzymes. This pLUTdNB vector is pTRIPZ base modified doxycycline-inducible expression vector. pLUTdNB plasmid ligated with E7 cDNA was then transformed into XL10-Gold Ultracompetent cells (Agilent Technologies, CA, USA) using β-mercaptoethanol to increase transformation efficiency, according to the manufacture's protocol. After transformation, the plasmid was isolated and then sequenced to confirm the cloning of full-length of HPV16 E7 cDNA into a pLUTdNB lentiviral expression vector.

Production of E7 overexpressing lentiviral particles: Lentivirus packaging protocol was followed as described by Applied Biological Materials Inc. (Richmond Canada) with some modifications. HEK 293T cells were co-transfected with the lentiviral constructs pVSV-G (envelope vector) and psPAX2 (packaging vector) (Addgene) along with a pLUTdNB plasmid

expressing full length of E7 (expression vector). Empty vector was used as a control and transfected into HEK 293T cells. Transfections were carried out using TurboFect Transfection Reagents in Opti-MEM® Reduced-Serum Medium (Thermo Fisher Scientific). Later, the culture medium was centrifuged at 3,000 rpm for 15 mins at 4°C. The filtered supernatant was used for lentiviral infection.

HPV16 E7 shRNA lentiviral particles: HPV 16 E7 shRNA lentiviral particles and control shRNA lentiviral particles were purchased from Santa Cruz Biotechnology, Inc, USA. HPV 16 E7 shRNA lentiviral particles contain a pool of concentrated, transduction-ready viral particles containing 2 target- specific constructs that encode 19-25 nucleotide shRNA designed to knock down gene expression. Control shRNA Lentiviral Particles encodes for a scrambled shRNA sequence suitable as a negative control for shRNA lentiviral transduction experiment.

Lentiviral particles transduction: A *p*rotocol for lentivirus particles transduction was followed as described by Santa Cruz Biotechnology, Inc. Before lentiviral transduction, the working concentration of puromycin was determined for the selection of transduced UMSCC-1 (HPV-negative) or UMSCC-47 (HPV-positive) cell lines. For this, in a separate experiment, UMSCC-1, and UMSCC-47 cells were treated with varying concentrations of puromycin dihydrochloride (Santa Cruz Biotechnology, Inc) from 1 µg/ml to10 µg/ml. Then, an optimal concentration of puromycin (2 µg/ml) was selected as the lowest concentration that kills 100% of non-transfected cells in 2-3 days.

For lentiviral infection, cells were seeded in a 12-well plate 24 hours prior to viral infection. Next day, media was replaced with Polybrene (Santa Cruz Biotechnology, Inc) containing media to enhance binding of pseudo-viral capsid to the cell membrane. Then, cells were infected with lentiviral particles (E7 over-expressing particles in UMSCC-1; and E7 shRNA lentiviral particles in UMSCC-104 cell lines). After cells underwent lentiviral transduction, stably transfected clones were selected using 2 µg/ml puromycin. After antibiotic selection, cells were expanded for stable E7 cDNA or E7 shRNA expression in UMSCC-1 and UMSCC-47 respectively. Cells stably expressing control shRNA or control empty vector was also isolated via puromycin selection.

E7 mRNA expression: Total RNA was isolated and cDNA was synthesized following the same protocol as described in chapter IV using primers against E7 by SYBR Green assay.

Western Blot Analysis: Cells were lysed and total protein was extracted using RIPA buffer (Cell Signaling Technology) and protease inhibitors as per the protocol. Total protein concentration was determined using the Bio-Rad Bradford protein assay reagent (Bio-Rad Laboratories, USA) with bovine serum albumin (BSA; Pierce, USA) as a standard. Extracted protein samples were mixed with sodium dodecyl sulfate (SDS) loading buffer (20% glycerol, 4% SDS, 100 mM Tris, pH 6.8, 0.002% bromophenol blue), and heated at 95°C for 5 min. These proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels (NUPAGE 10% Bis-TRIS, Invitrogen, USA) and transferred to polyvinylidene difluoride (PVDF) membrane (Immobilon-P transfer membrane, EMD Millipore, USA), which were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TBST) for 1 h at room temperature. The membrane was then incubated with mouse monoclonal anti-E7 antibody (Abcam / Santa Cruz Biotechnology, USA) or rabbit polyclonal anti-β-actin (Genetex) antibodies overnight at 4 °C. After washing with TBST for 3 times, membranes were incubated with 1:5000 diluted secondary antibodies [horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG or HRP-conjugated mouse anti-rabbit IgG (Thermo Scientific Pierce, USA)] for 1 h at room temperature. Proteins were detected by chemiluminescence (SuperSignal West Dura Extended Duration Substrate; Thermo Scientific Pierce, USA) and bands were visualized on X-ray film (CLXPosure Film, Thermo Scientific Pierce, USA) using an SRX-101A processor (Konica Minolta Medical Imaging USA, Inc).

Results and Discussion

Cloning of E7 into lentiviral expression vector

HPV16 E7 cDNA was amplified and cloned into a pLUTdNB lentiviral expression vector (**Figure S2**). After cloning, isolated plasmid was digested using *Eco*RI and *Xho*I, which gave ~300bp product in an agarose gel, which corresponded to the full length of HPV16 E7 (**Figure S2-D**). For verification, the sequence of cloned vector was compared with other sequence variants of HPV16 (such as AF402678.1, AF125673.1, AY686584.1, NC_001526.2,

and KF954093.1) using SeqMan Pro program (Lasergene 12, DNAstar Inc., Wisconsin) and NCBI BLASTN database. The obtained full-length nucleotide sequence was then translated into an amino acid sequence using EditSeq (DNASTAR) software and compared with other sequences using NCBI BLASTP database. 100% sequence identity was obtained between the cloned sequence with protein sequences of HPV16 E7 (accession no. AAO85409.1), suggesting the successful cloning of HPV16 E7 cDNA into lentiviral expression vector pLUTdNB.

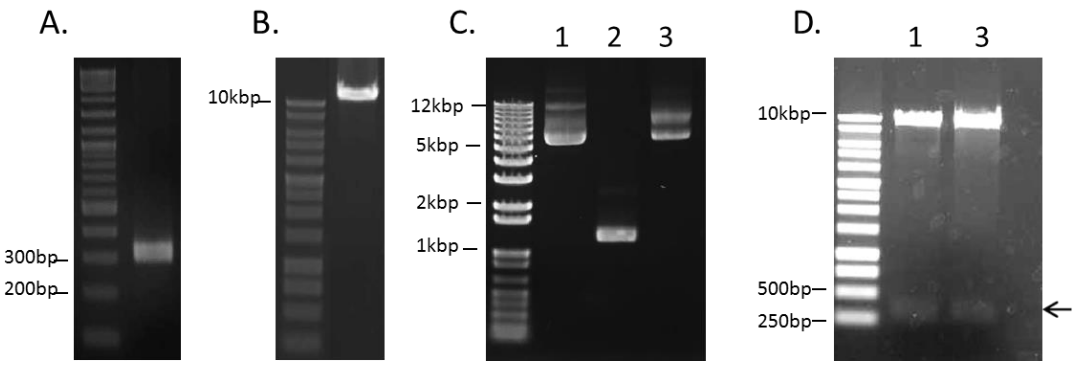

Figure S2: Cloning of E7 into a pLUTdNB lentiviral expression vector. **A)** Gel image showing *Eco*RI and *Xho*I digested E7 DNA, which was initially PCR-amplified from HPV16 plasmid. This DNA band was purified from the gel and used to ligate with digested pLUTdNB. **B)** Gel image showing *Eco*RI and *Xho*I digested pLUTdNB vector, which was purified from the gel. Restriction digested E7 and pLUTdNB DNAs were ligated and cloned into *E. coli*. **C)** Gel image showing cloned E7-pLUTdNB plasmid DNAs (Lanes #1 and #3) isolated from transformed *E. coli*. (Lane #2 showed the absence of cloned plasmid) **D)** Cloned plasmids were digested with *Eco*RI and *Xho*I that produced the fragments of ~300 bp (shown by the arrow), which corresponded to the full-length of E7.

Lentiviral knockdown of E7 in HPV-positive HNC cell line

After lentiviral transduction, puromycin selection was done to obtain UMSCC-47 cells stably expressing E7 shRNA. Puromycin-resistant cells were obtained, indicating successful lentiviral transduction. However, as shown **in Figure S3**, knockdown of E7 did not significantly reduce E7 mRNA in UMSCC-47 cells as compared to cells stably expressing scrambled control shRNA. To confirm above results, E7 protein levels was tested by

immunoblotting, but could not be detected. Even a positive control (*i.e.* purified E7 protein) was not detectable in the immunoblot. This may be due to the low quality of commercial E7 antibodies. However, the immunoblot was positive for house-keeping protein (β- actin). So, further experiments have to be done to obtain effective knockdown of E7 in HPV-positive UMSCC-47 cell line.

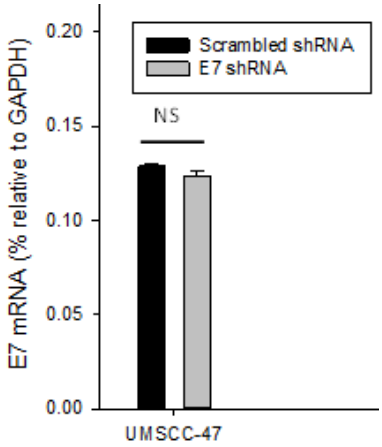

Figure S3: E7 expression in UMSCC-47 cells after the lentiviral shRNA-mediated knockdown, showing unsuccessful knockdown of E7 compared to scrambled control shRNA.

Lentiviral transduction of E7 cDNA in HPV-negative HNC cell line

E7 overexpressing lentiviral particles were produced and then transduced in HPV-negative UMSCC-1 cell line. However, transduced cells started dying after treatment with 2 µg/ml puromycin (similar to non-transduced cells), indicating unsuccessful lentiviral transduction. This may be due to the poor quality of lentiviral particles or due to other reasons. So, experiments should be repeated troubleshooting the relevant issues.

III. Epigenetic changes in host tumor suppressor genes *SMG1*, *RUNX3,* and *CHFR* in HNCs

In chapter V, I selected 7 genes (*GRB7*, *RUNX3*, *RUNX2*, *CHFR*, *RARB, EREG,* and *SMG-1*) from 38 tumor suppressors as the targets for further studies and then focused on the promoter methylation status of *GRB7* and *EREG,* since they were the potential candidate genes regulated in HPV-positive HNC cell lines. I have not included the promoter methylation status of other genes as not to distract from the focus of the project, therefore have discussed in this part of the appendix.

Correlation of DNA methylation status of SMG1, RUNX3 and CHFR promoters with their expression

The methylation status of promoters of *SMG1*, *RUNX3* and *CHFR* and the effect of de-methylation treatment were analyzed using MS-PCR method. Both HPV-positive (UMSCC-47) and HPV-negative (UMSCC-1) HNC cell lines contained methylated as well as a unmethylated *SMG1* promoter (**Figure S4 A-B**). *RUNX3* and *CHFR* methylation status was detected in both cell lines (**Figure S4C-F**). Even on de-methylation treatment, there was no change in bands of all three genes- *SMG1*, *RUNX3,* and *CHFR* (**Figure S4**). These results suggest that epigenetic regulations of *SMG1*, *RUNX3* and *CHFR* may not be specific to HPV-positive HNCs.

Expressions of *SMG1*, *RUNX3*, and *CHFR* genes were higher in UMSCC-1 than UMSCC-47 (**Figure 28**), but promoter methylation status was found similar in both cell types (**Figure S4**). This result indicated that in the case of these 3 genes, the correlation between increased promoter methylation and decreased expression tended to be low. There was also no change in methylation status of the promoter of these genes even after de-methylation treatment (**Figure S4**), although 5-aza-dc caused increased *SMG1* expression in UMSCC-1, and increased expression of *RUNX3* and *CHFR* in both cell types (**Figure 31**).These results additionally indicate that methylation-mediated gene silencing of *SMG1*, *RUNX3*, and *CHFR* may not be cell-type specific.

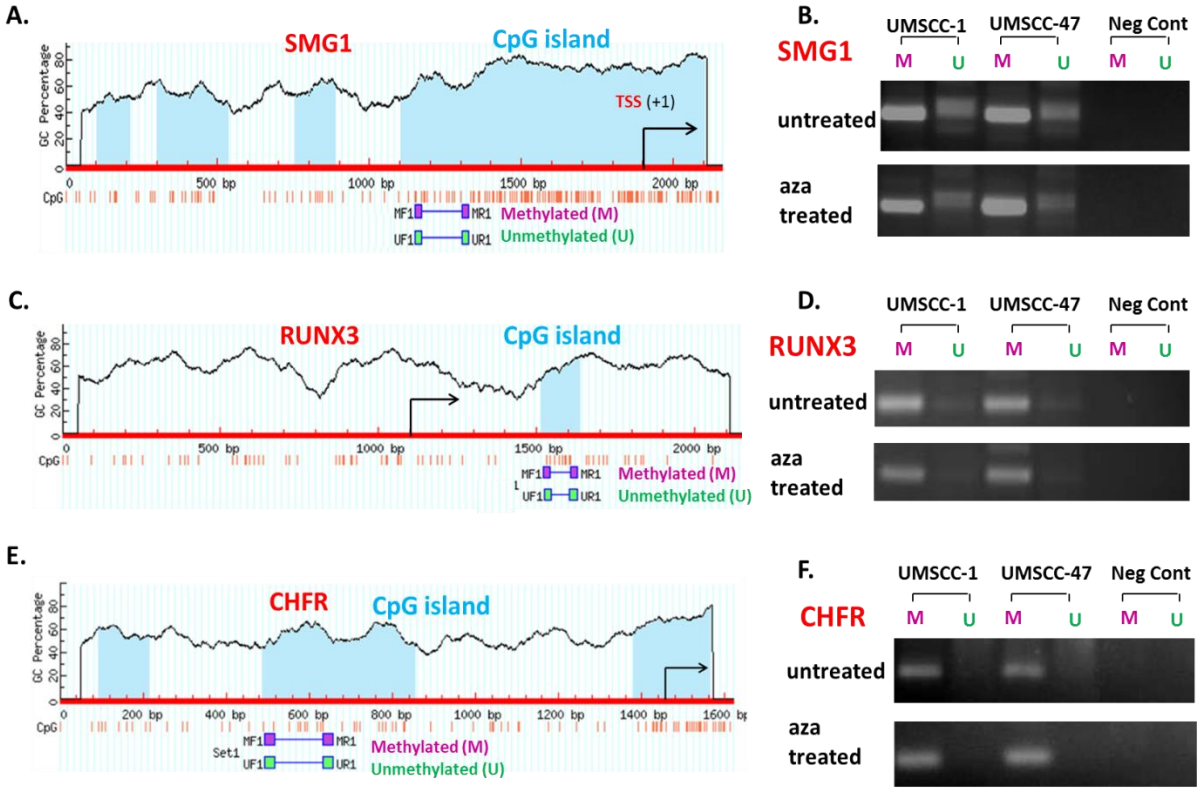

Figure S4: Methylation status of *SMG1*, *RUNX3,* and *CHFR* promoters. Promoter regions of *SMG1* **(A)**, *RUNX3* **(C)** and *CHFR* (**E)**, showing CpG island and the locations of methylation-specific PCR (MS-PCR) primers. Agarose gel images showing MS-PCR of *SMG1* **(B)**, *RUNX3* **(D)** and *CHFR* **(F)** in bisulfite-modified DNA from HPV-negative (UMSCC-1) and HPV-positive (UMSCC-47) cell lines with or without 5-aza-dc (5 µM for 96 hours) treatment. No template DNA serves as negative control.

LIST OF ABBREVIATIONS AND SYMBOLS

5-aza-dc	5-aza-2′-deoxycytidine
CIN	Cervical intraepithelial neoplasia
CpG	5'- Cytosine-phosphate-Guanine-3'
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
E2BS	E2 binding sites
E2F-TF	E2 factor family of transcription factors
EGFR	Epidermal growth factor receptor
EREG	Epiregulin
FFPE	Formalin-fixed, paraffin-embedded
hgOED	High-grade oral epithelial dysplasia
HN	Head and Neck
HNC	Head and Neck carcinoma/ cancers
HNSCC	Head and Neck squamous cell carcinoma
HPV	Human papillomavirus
H&E	Hematoxylin and eosin staining
IHC	Immunohistochemistry
ISH	*in situ* hybridization
LCR	Long control region
MEH	Multiple epithelial hyperplasia
MS-PCR	Methylation-specific PCR
OCSCC	Oral Cavity Squamous Cell Carcinoma
OPSCC or OPC	Oropharyngeal Squamous Cell Carcinoma
OPC	Oropharyngeal cancer
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
PV	Papillomavirus
qRT-PCR	Quantitative Reverse Transcription-PCR
Rb	Retinoblastoma
SCC	Squamous Cell Carcinoma
SNPs	Single nucleotide polymorphisms
TBP	TATA binding protein
TSG(s)	Tumor suppressor gene(s)
URR	Upstream Regulatory Region

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GRANTS / FELLOWSHIP

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(http://louisville.edu/codre/codre-publications/newsletters-1/codre-newsletter-spring-2016)

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Internal grant for oral and oropharyngeal cancer research July 2014- Aug 2016
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PUBLICATIONS

1. **Khanal, S.**, Cole, E. T., Joh, J., et al. (2015). Human papillomavirus detection in histologic samples of multifocal epithelial hyperplasia: a novel demographic presentation. *Oral Surg Oral Med Oral Pathol Oral Radiol, 120*(6), 733-743.
   - Poster abstract published in Sep 2015 http://www.oooojournal.net/article/S2212-4403(15)00991-8/abstract

2. **Khanal, S.**, Joh, J., Kwon, A. M., et al. (2015). Human papillomavirus E7 serology and association with p16 immunohistochemistry in squamous cell carcinoma of the head and neck. *Exp Mol Pathol, 99*(2), 335-340.

3. **Khanal, S.***, Ferraris, E. D., Zahin, M., Joh, J., Ghim, S. J., & Jenson, A. B. (2015). Targeting synthetic Human Papillomavirus (HPV) L2 disulfide-induced N-terminus conformational epitopes for pan-HPV vaccine development. *Exp Mol Pathol, 99*(2), 330-334. (***corresponding author)**
   - Paper featured in *World Biomedical Frontiers* http://biomedfrontiers.org/cancer-2016-3-3/

4. Moore, A. R., Libby, A. L., **Khanal, S.**, Ehrhart, E. J., 3rd, & Avery, P. (2015). Is this cell hollow? *Vet Clin Pathol*.

5. Zahin, M., Ghim, S. J., **Khanal, S.**, Bossart, G. D., Jenson, A. B., & Joh, J. (2015). Molecular characterization of novel mucosotropic papillomaviruses from a Florida manatee (*Trichechus*

manatus latirostris). *J Gen Virol*. 96 (12), 3545-3553

6. **Khanal S.**, Trainor PJ., Zahin M., Ghim SJ., Joh J., Rai SN., Jenson A., Shumway BS. Histologic variation in high grade oral epithelial dysplasia when associated with high-risk human papillomavirus. (*Oral Surg Oral Med Oral Pathol Oral Radiol*, Submitted, under Revision)
    - Poster abstract published in Feb 2016 in *International Journal of Radiation Oncology • Biology • Physics, 94*(4), 944. http://dx.doi.org/10.1016/j.ijrobp.2015.12.277

7. Perez CA, Amsbaugh MJ,...**Khanal S**,…. Redman, R. High-Dose Versus Weekly Cisplatin Definitive Chemoradiation Therapy for Human Papillomavirus–Related Oropharyngeal Squamous Cell Carcinoma of the Head and Neck (2016). Poster abstract published in *International Journal of Radiation Oncology • Biology • Physics, 94*(4), 895. http://dx.doi.org/10.1016/j.ijrobp.2015.12.097

8. Zahin M., Joh J., **Khanal S.**, Husk A., Mason H., Warzecha H., Ghim SJ., Miller D.M., Matoba N., Jenson A. Scalable Production of HPV16 L1 Protein and VLPs from Tobacco Leaves. (*PLOS ONE* Submitted, under Revision)

9. Vatsalya V, **Khanal S.**, Jenson A., Srivastava S., Schwandt M., Ramchandani V.A. Role of linoleic acid in the exacerbation of liver injury in treatment naïve HIV-diagnosed alcohol dependent patients. (*AIDS Research and Human Retroviruses* Submitted, under Review)

AWARDS / SCHOLARSHIPS

University Topper Merit Award, *Jiwaji University*, M.P, India — 2011

College Topper Award, *Boston College for Professional studies*, M.P, India — 2011

Half **Tuition Waiver**, *Boston College for Professional studies*, India — 2009-2011
(Scoring above 80% in every semester, M.Sc. Biochemistry)

Award of Excellence, *Boston College for Professional studies*, India — 2009-2011
(**First Position,** creating decorative items from waste materials)

University Topper Award, *Purbanchal University*, Nepal — 2007

Full **Tuition Waiver**, *National College for Advance Learning*, Nepal — 2003-2007
(**Highest Rank** in every semester, B. Sc. Biochemistry)

Graduation Award, *National School of Sciences*, Nepal — 2002
(**Distinction** status, Higher Secondary Board Examination)

Tuition Waiver, *National School of Sciences*, Nepal — 2000-2002
(**Highest Rank** among female students, Higher Secondary Grade 11 & 12)

Aawhan Samuha S.L.C. Award, Aawhan Samuha, Kathmandu, Nepal — 2000
(Fourth among S.L.C. students of Kathmandu Metropolitan City, Ward # 7)

Tanka Bdr. Pradhan Memorial Medal, *Baba Boarding High School*, Nepal — 2000
(**Silver Medal**, Second among the S.L.C. candidates)

Tuition Waiver, *Baba Boarding High School*, Nepal — 1998-2000
(Second position, scoring above 80% in final exams)

Peer Reviewer of Manuscripts

Currently serving as a peer reviewer for Cancer Informatics of Libertas Academica

Poster Presented at National and International Meetings

- Multidisciplinary Head and Neck Cancer Symposium, *Scottsdale, Arizona* — Feb 18-20, 2016
- 30th International Papillomavirus Conference & Clinical Workshop (HPV 2015), *Lisbon, Portugal* — Sep 17-21, 2015

- Joint Annual Meeting of American Academy of Oral and Maxillofacial Pathology/American Academy of Oral Medicine, San Diego, *California* April 18-24, 2015
- Postdoctoral Research Symposium, *University of Kentucky*, KY June 12, 2015
- Annual James Graham Brown Center Retreat, *University of Louisville* Oct 2013,Oct 2014
- Research! Louisville, *University of Louisville* Oct 2013,Sep 2014
- Graduate Research Symposium, *University of Louisville* Nov 2014

ORAL PRESENTATIONS

1. Dissertation Defense, Department of Biochemistry and Molecular Genetics
   University of Louisville, Louisville, KY July 15, 2016
   "Molecular Characterization of Human Papillomavirus in Head and Neck Tumors"

2. James Graham Brown Cancer Center Colloquia
   University of Louisville, Louisville, KY Apr 6, 2016
   *"*Human Papillomavirus in Head and Neck Tumors"

3. Three Minute Thesis Competition (Founded by the University of Queensland)
   2016 Research Conference, *University of Louisville, Louisville, KY* Apr 1, 2016
   "Histologic variation in high-grade oral epithelial dysplasia when associated with HPV"

4. Commission on Diversity and Racial Equality (CODRE) meeting
   University of Louisville, Louisville, KY Jan 14, 2016
   "Human Papillomavirus Detection in Histological Samples of Premalignant Oral Lesions"

5. Research Conference, Department of Biochemistry and Molecular Genetics
   University of Louisville, Louisville, KY Oct 28, 2015
   "Molecular Characterization of Human Papillomavirus in different lesions of Head and Neck"

6. Seminar, Department of Biochemistry and Molecular Genetics
   University of Louisville, Louisville, KY Feb 23, 2015
   *"*Current Insights into HPV Integration-Driven Carcinogenesis"

POSTER PRESENTATIONS

1. **Khanal S**, Shumway BS, Joh J, Zahin M, Ghim SJ, Redman R, Bumpous J, Jenson AB "Human papillomavirus (HPV) prevalence and HPV DNA integration status in benign, premalignant and cancerous lesions of the head and neck"
   - Research Retreat –Department of Biochemistry and Molecular Genetics, August 21, 2015, *University of Louisville*, Louisville, USA
   - Research! Louisville, Oct-2015, *University of Louisville*, Louisville, KY

2. **Khanal S**, Trainor P, Rai SN, Zahin M, Ghim SJ, Joh J, Jenson AB, Shumway BS. "Histologic Variation in High-Grade Oral Epithelial Dysplasia when associated with High-Risk Human Papillomavirus"
   - 30th International Papillomavirus Conference & Clinical Workshop (HPV 2015), September 17-21, Lisbon, *Portugal*
   - 2016 Multidisciplinary Head and Neck Cancer Symposium, which will be held February 18-20 in *Scottsdale, AZ.*

3. **Khanal S**, Cole ET, Joh J, Ghim SJ, Jenson A, Rai SN, Trainor PJ, Shumway BS. "Human Papillomavirus Detection in Histological Samples of Multifocal Epithelial Hyperplasia: a novel demographic presentation"
   - Joint Annual Meeting of American Academy of Oral and Maxillofacial Pathology (AAOMP)/ American Academy of Oral Medicine (AAOM), April 18-24, 2015, San Diego, *California*. http://www.oooojournal.net/article/S2212-4403(15)00991-8/abstract
   - Postdoctoral Research Symposium, June 12, 2015, *University of Kentucky*, KY

4. **Khanal S**, Joh J, Redman R, Bumpous J, Ghim SJ, Jenson AB "Correlation of HPV serology with p16, smoking status and treatment response in oropharyngeal squamous cell carcinoma patients".
    - Research! Louisville, Sept-2014, *University of Louisville*, Louisville, KY
    - 14th James Graham Brown Center Retreat, Oct 17, 2014,*University of Louisville*, Louisville, KY
    - Graduate Research Symposium, Nov-2014, *University of Louisville*, Louisville, KY

5. **Khanal S**, Ghim SJ, Davis KR, Ellis S., and Jenson AB. "A soybean peptide-lunasin suppresses BPV-1 induced fibrosarcoma in mammalian cells"
    - Research! Louisville, Oct-2013, *University of Louisville*, Louisville, KY
    - 13th Annual James Graham Brown Center Retreat Oct 25, 2013, *University of Louisville*

6. Zahin M, Ghim SJ, **Khanal S**, Jenson AB, Joh J. "Molecular characterization of two novel mucosotropic papillomaviruses isolated from a Florida Manatee (*Trichechus manatus latirostris)*".
    - 14th James Graham Brown Center Retreat, Oct 17, 2014,*University of Louisville*, Louisville, KY

7. Zahin M, Joh J, **Khanal S**, Husk A, Mason H, Ghim SJ, Matoba N, Jenson AB. "Plant-based Recombinant Vaccine: Purification of HPV16 L1 Protein And VLPs from Tobacco Leaves"
    - PBVAB (Plant-Based Vaccines, Antibodies & Biologics) June 8-10 2015, Lausanne, *Switzerland*
    - 30th International Papillomavirus Conference & Clinical Workshop (HPV 2015), September 17-21, Lisbon, *Portugal*

8. Perez CA, Wu X, Amsbaugh MJ, Claudino WM, Yusuf M, Roberts T, Rios-Perez JA, Jain D, Jenson A, **Khanal S**, Silverman CI, Tennant P, Dunlap NE, Rai S, Redman R. "High-dose versus weekly cisplatin definitive chemoradiotherapy for HPV-related oropharyngeal squamous cell carcinoma of the head and neck"
    - 2016 ASCO (American Society of Clinical Oncology) Annual Meeting, June 3-7, 2016, Chicago, Illinois
      https://asco.confex.com/asco/2016/sci/papers/viewonly.cgi?username=166554&password=766578
      http://www.redjournal.org/action/doSearch?searchType=quick&searchText=khanal&occurrences=all&journalCode=rob&searchScope=fullSite

RESEARCH EXPERIENCE

Pre-doctoral research and other lab projects Jan 2013- Aug 2016

Dr. Alfred Bennett Jenson and Dr. Steven R. Ellis (Supervisors), University of Louisville, KY
Dr. Jenson's Lab, James Graham Brown Cancer Center, University of Louisville

- Molecular characterization of HPV-associated tumors of the head and neck, such as studies on HPV DNA Integration, Methylation, Virus-induced epigenetic regulation and Fanconi Anemia-DNA repair Pathway
- HPV-serotyping and estimation of antibody titer in sera of head and neck cancer patients
- HPV detection in histological samples from benign and premalignant oral mucosal lesions
- Animal (Cat, Dog, Macaque, Manatee) papillomavirus infections
- Plant-based HPV vaccine development
- Determination of the effects of lunasin on BPV-1 induced fibrosarcoma in NIH 3T3 cells
- Papillomavirus isolation and purification by cesium chloride ultracentrifugation method

Lab Rotation Sep-Dec, 2012

Dr. Schaner Tooley's Lab, University of Louisville, KY

- Determine the effects of NRMT loss and/or inhibition of primary mammary epithelial cell growth
- Mouse mammary gland dissection, production of primary epithelial cells for culture, lentivirus production, cell growth assays and immunofluorescence techniques

M.Sc. Dissertation March-July, 2011

"Khanal, S., and Sharma, P.R. (2011) Anti-cancer activity of Withaferin A, an active compound from *Withania somnifera*: Induction of apoptosis and inactivation of PI3K pathway. M.Sc. in Biochemistry Dissertation, Indian Institute of Integrative Medicine, Council of Science & Industrial Research, Jammu, India"

- Studied anti-cancer activity of withaferin A (WA, an active compound from *Withania somnifera)* using scanning electron microscopy and fluorescent microscopy.

- Anti-cancer studies on WA-treated cancer cells included determination of apoptotic index, mitochondrial membrane potential studies, immune-fluorescent studies of PI3K/AKT pathway, ultra-structural analysis for apoptotic phenotypes.
- Sample preparation for electron microscopy using ultramicrotome and sputter coater

B.Sc. Dissertation July- Oct 2007

"Khanal, S., Pradhan, M. M., and Subedi, S. (2007). Study on the effect of physiological, nutritional and clinical parameters on serum uric acid level in patients suspected of gout. B.Sc. in Biochemistry Dissertation, National College of Advance Science, Medi-Clinic Pvt. Ltd., Kathmandu, Nepal"

- Measurement of serum uric acid level by uricase method and statistical studies to compare serum uric acid levels of asymptomatic hyperuricemic patients with normouricemic patients and with patients having gouty attacks and their variation in terms of age, gender, nutritional status, alcohol intake, smoking habit, family disposition, and relevant pathological condition

Internship July- Oct 2007

Medi-Clinic Pvt. Ltd., Kathmandu, Nepal

- Conducted routine laboratory works at Biochemistry, Hematology, Immunology, Parasitology and Microbiology Departments
- Learned patient sample (blood, oral / wound swab) collection techniques
- **Biochemical tests** of patient samples such as Blood Glucose, Uric acid, Lipid profile, Liver function test, Renal function test, Thyroid function test, Cardiac function test
- **Hematological tests:** Total Leucocyte Count, Differential Leucocyte Count, RBC Count, Platelet Count, Blood Grouping, Cross Matching, Hemoglobin Estimation, Bleeding and Clotting time, Prothrombin Time, ESR, PCV, Hematocrit, HBA_1C test
- **Immunological tests**: Monteux, WIDAL, VDRL, CRP, RPR, ANF, TPA, HIV tests
- **Microbiological tests**: Analysis of patient's blood, urine, and stool.

WORK EXPERIENCE

Graduate Research Assistant July 1, 2014- present

James Graham Brown Cancer Center, University of Louisville, KY, USA

Teaching Assistant Jan- Apr 2014

Advanced Biochemistry-II (BIOC 647) under Dr. William L. Dean
Dept. of Biochemistry & Molecular Biology, University of Louisville, KY, USA

Biochemist Apr 2008- Mar 2009

B.P. Smriti Hospital, Kathmandu, Nepal
Undertook different pathological test in clinical chemistry

Volunteer Dec, 2007- Feb, 2008

Medi-Clinic Pvt. Ltd., Kathmandu, Nepal
Assisted and Undertook Biochemical Tests in Pathological Laboratory

Instructor Apr, 2007- Nov, 2008

Hi-Tech Educational Institute**,** Kathmandu, Nepal,
Taught Mathematics and Science courses to Secondary and Higher Secondary Students

MEMBERSHIP

Vice-President, *Nepalese Student Association* (NSA),
University of Louisville, Louisville, KY — Jan 2015- Aug 2016

Member, *American Medical Student Association*, Sterling, Virginia — May 2014- present

Member, *The Kentucky Academy of Science*, Louisville, KY — Jan 2014- present

Secretary, *Nepalese Student Association* (NSA),
University of Louisville, Louisville, KY — Jan 2013- 2014

Member, *American International Relations Club* (AIRC)
University of Louisville, Louisville, KY — Dec 2012- present

PROFESSIONAL INVOLVEMENT

Completed **Flow Cytometry Bootcamp** — August 4-5, 2014
Organized by Excyte Leaders and DOC Flow Cytometry Core
University of Louisville, Louisville, KY

Attendee, CGeMM Symposium "Genomics in Medicine" — 2014 and 2015
Organized by Center for Genetics and Molecular Medicine,
Held at University of Louisville, Louisville, KY

Attendee, Speed Networking "A career networking event" — April 24, 2014
Organized by Office of Graduate and Postdoctoral Studies
Held at University of Louisville, Louisville, KY

Completed **Professional Grant Development Workshop** — June 26-28, 2013
Organized by Grant Training Center, Arlington, VA
Held at University of Louisville, Louisville, KY

Attendee, Symposium "Technologies in Carcinogenesis & Chemoprevention"
Held at University of Louisville, Louisville, KY — May 30-31, 2013

Volunteer, "2016 Cancer Survivors day" — May 05, 2016
James Graham Brown Cancer Center and Kentucky Cancer Program

REFERENCES

Alfred Bennett Jenson, MD
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James Graham Brown Cancer Center
University of Louisville, KY, USA
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alfred.jenson@louisville.edu

Steven R. Ellis, Ph.D.
Professor
Dept. of Biochemistry and Molecular Genetics
University of Louisville, KY, USA
Phone: 502-852-5222
steven.ellis@louisville.edu