HPV AND ORAL INNATE IMMUNITY: A ROLE FOR E5

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ABSTRACT

Thatsanee Saladyanant: HPV and Oral Innate Immunity: A Role for E5
(Under the direction of Jennifer Webster-Cyriaque)

High (HR) and low-risk (LR) Human Papillomaviruses (HPVs) are known as the causative agents for benign and malignant lesions in the head and neck region. HR-and LR-infections differentially modify signal transduction and host immune responses. Of the HPV oncoproteins, HPVE5 is a small hydrophobic protein whose role is undefined in oral keratinocytes. We hypothesized that HR and LR-HPVE5 proteins differentially impact oral signal transduction, type I interferon responses, and cell differentiation processes. Our long-term goal is to elucidate the mechanisms of HPV integration. We generated pCMV plasmid vectors containing; whole genomes with E5 (HR-HPV16WT) or with E5 deleted (HPV16ΔE5), HPV16E5-tagged, transcribed but un-translated HPV16E5 (in the context of the whole genome or overexpressed), and LR-HPV6E5 genome. All constructs were transfected into oral keratinocytes and human primary foreskin keratinocytes. By growing cells on monolayer system, higher expression of phospho-p38 MAPK, phospho-MEK1/2 and phospho-Erk1/2 proteins were detected with HR-HPV16E5 but not with LR-HPV6E5 over-expression. Phospho-AKT and phospho-JNK levels were not different between HR and LR. The HR-E5 mRNA maintained MAPK activation implying a role for E5 transcripts in MAPK modulation. This effect was diminished in the absence of EGF and in the presence of terminal differentiation. HR-HPV16E5 demonstrated higher interferon promoter activity and ISG expression (ISG54 and 2’5’ OAS) that was inhibited with Cox-2 inhibitor. The HPV16ΔE5 behaved similarly to LR-HPV6E5.
HPV16E5 also showed the suppressive effect on E6 splicing. By growing cells in organotypic raft culture, HPV16WT and HPV16E5 induced hyper-proliferation, activated spinous differentiation, and delayed terminal differentiation. HPV16E5 induced higher STAT1, and STAT3 expression compared to HPV6E5. In conclusion, cell proliferation and activation of intracellular immune responses are critical to transformation of oral keratinocytes and subsequent cancer development. HR-HPV16E5 increased MAPK proliferation signals, enhanced type I interferon responses and STAT3, delayed terminal differentiation, enhanced spinous differentiation, and control E6 and E7 expression whereas LR-HPV6E5 and HPV16ΔE5 barely did. In summary, HR-HPV16E5 may be a key facilitator of oncogenic progression in the head and neck region while LR-HPV6E5 is not.
To mom and dad who are always by my side—no matter how I am.
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LIST OF ABBREVIATIONS

AAP  American Academy of Periodontology
AIDS  Auto Immune Deficiency Syndrome
AIM  Absence of Melanoma
AKT  RAC-alpha serine/threonine-protein kinase
ASC  Apoptosis Associated Speck-like Protein Containing a Caspase Recruitment Domain
ATP  Adenosine Triphosphate
BGI  Biofilm Gingival Index
BI  Bleeding Index
BOP  Bleeding on Probing
BPE  Bovine Pituitary Enzyme
CaCl  Calcium Chloride
CARD  Caspase Activation and Recruitment Domain
CD  Cluster of Differentiation
CIN  Cervical Intraepithelial Neoplasia
CMV  Cytomegalovirus
COPV  Canine Oral Papillomavirus Infection
Cox  Cyclooxygenase
CpG  Cytidine-phosphate-Guanosine
CRP  C-Reactive Protein
CXCL10  Interferon-gamma Inducible Protein 10 kDA
DAI  DNA-dependent activator of interferon regulator factors
DAPI  4′6′-diamidino-2-phenylindole
DNA  Deoxyribonucleic Acid
ds  Double-strand
EGF  Epithelial Growth Factor
EGFR  Epithelial Growth Factor Receptor
ER  Endoplasmic Reticulum
ERK  Extracellular Signal-regulated Kinase
FADD  Fas-associated Protein with Death Domain
FAS  First Apoptosis signal
FBS  Fetal Bovine Serum
FGFR  Fibroblast Growth Factor Receptor
GAPDH  Glyeraldehyde 3-phosphate Dehydrogenase
GAS  IFN-gamma Activated Site
GCF  Gingival Crevicular Fluid
GI  Gingival Index
HA  Haemagglutinin
HAART  Highly Active Antiretroviral Therapy
HFK  Human Foreskin Keratinocyte
HIV  Human Immunodeficiency virus
HLA  Human Leukocyte Antigen
HNSCC  Head and Neck Squamous Cell Carcinoma
HPV  Human Papillomavirus
HRSA  HIV Oral Health Demonstration Project
IFA  Immunofluorescence assay
IFI  Interferon Inducible Gene
IFN  Interferon
IgA  Immunoglobulin A
<table>
<thead>
<tr>
<th>Term</th>
<th>Description</th>
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<tbody>
<tr>
<td>IKK</td>
<td>IκB kinases</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IPS-1</td>
<td>Interferon Promoter Stimulator-1</td>
</tr>
<tr>
<td>IRAK</td>
<td>IL-1 receptor associated kinase</td>
</tr>
<tr>
<td>IRF</td>
<td>Interferon Regulatory Factor</td>
</tr>
<tr>
<td>ISG</td>
<td>Interferon Stimulated Gene</td>
</tr>
<tr>
<td>ISRE</td>
<td>Interferon Stimulated Regulatory Element</td>
</tr>
<tr>
<td>Jak</td>
<td>Janus Kinase</td>
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<tr>
<td>JNK</td>
<td>c-Jun N-terminal Kinase</td>
</tr>
<tr>
<td>K1</td>
<td>Keratin 1</td>
</tr>
<tr>
<td>K10</td>
<td>Keratin 10</td>
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<tr>
<td>Kb</td>
<td>Kilo Basepair</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>KGFR</td>
<td>Keratinocyte Growth Factor Receptor</td>
</tr>
<tr>
<td>LCR</td>
<td>Long Control Region</td>
</tr>
<tr>
<td>luc</td>
<td>Luciferase</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
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<tr>
<td>MAPK</td>
<td>Mitogen Activated Protein Kinase</td>
</tr>
<tr>
<td>MCP</td>
<td>Methyl-accepting chemotaxis protein</td>
</tr>
<tr>
<td>MDA</td>
<td>Melanoma Differentiation Associated Gene</td>
</tr>
<tr>
<td>MEK</td>
<td>Mitogen-activated Protein Kinase Kinase</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
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<tr>
<td>MKK</td>
<td>Mitogen-activated Protein Kinase Kinase</td>
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<tr>
<td>mM</td>
<td>Milli Molar</td>
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<tr>
<td>MNDA</td>
<td>Myeloid Cell Nuclear Differentiation Antigen</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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<tr>
<td>MyD88</td>
<td>Myeloid Differentiation Primary response Gene88</td>
</tr>
<tr>
<td>NaB</td>
<td>Sodium Butyrate</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear Factor Kappa-light-chain-enhancer of Activated B Cells</td>
</tr>
<tr>
<td>ng</td>
<td>Nano gram</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer cell</td>
</tr>
<tr>
<td>NLR</td>
<td>NOD Like Receptor</td>
</tr>
<tr>
<td>NLRP</td>
<td>Nucleotide-binding Oligomerization Domain, Leucine-rich Repeat and Pyrin Domain Containing Protein</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide-binding Oligomerization Domain</td>
</tr>
<tr>
<td>NOK</td>
<td>Normal Human Oral Keratinocyte</td>
</tr>
<tr>
<td>OAS</td>
<td>Oligoadenylate Synthetase</td>
</tr>
<tr>
<td>OHARA</td>
<td>Oral HIV and AIDS Research Alliance</td>
</tr>
<tr>
<td>OKF</td>
<td>Oral Keratinocyte</td>
</tr>
<tr>
<td>OPSCC</td>
<td>Oropharyngeal Squamous Cell Carcinoma</td>
</tr>
<tr>
<td>ORF</td>
<td>Open Reading Frame</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen Associated-Molecular Pattern</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PD</td>
<td>Pocket Depth</td>
</tr>
<tr>
<td>poly I:C</td>
<td>Polyinosinic:polycytidylic acid</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern Recognition Receptor</td>
</tr>
<tr>
<td>q-PCR</td>
<td>Quantitive Polymerase Chain Reaction</td>
</tr>
<tr>
<td>Rb</td>
<td>Retinoblastoma</td>
</tr>
<tr>
<td>RIG-I</td>
<td>Retinoic Acid Inducible Gene I</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RRP</td>
<td>Recurrent Respiratory Papilloma</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<td>------------</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse Transcriptase Polymerase Chain Reaction</td>
</tr>
<tr>
<td>sh</td>
<td>Short Hair Pin</td>
</tr>
<tr>
<td>SLPI</td>
<td>Secretory Leukocyte Peptidase Inhibitor</td>
</tr>
<tr>
<td>ss</td>
<td>Single-strand</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal Transducer and Activator of Transcription</td>
</tr>
<tr>
<td>STING</td>
<td>Stimulator of Interferon genes</td>
</tr>
<tr>
<td>TAK</td>
<td>TNF-related Activation Protein Associated Kinase</td>
</tr>
<tr>
<td>TANK</td>
<td>TRAF Family Member-associated NF-kappa-B Activator</td>
</tr>
<tr>
<td>TAP</td>
<td>Transporter-associated with Antigen Processing</td>
</tr>
<tr>
<td>TBK</td>
<td>TANK-binding Kinase</td>
</tr>
<tr>
<td>TERT</td>
<td>Telomerase Reverse Transcriptase</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like Receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF Receptor Associated Factor</td>
</tr>
<tr>
<td>TRAP</td>
<td>TNF-related Activation Protein</td>
</tr>
<tr>
<td>Tyk</td>
<td>Tyrosine Kinase</td>
</tr>
<tr>
<td>UNC</td>
<td>University of North Carolina at Chapel Hill</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endoepithelial Growth Factor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular Endoepithelial Growth Factor Receptor</td>
</tr>
<tr>
<td>VL</td>
<td>Viral Load</td>
</tr>
<tr>
<td>WT</td>
<td>Wild Type</td>
</tr>
<tr>
<td>μM</td>
<td>Micro Molar</td>
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CHAPTER 1: HUMAN PAPILLOMAVIRUSES AND OROPHARYNGEAL DISEASES

Introduction

Head and neck squamous cell carcinoma (HNSCC) is associated with major morbidity worldwide. HNSCC is the sixth most prevalent cancer and the number of HNSCC cancer cases has risen to greater than 405,000 new cases per year and 200,000 deaths per year (1). Oral cavity cancer causes an estimated 263,900 new cases and 128,000 deaths each year (1, 2). Major risk factors for HNSCC include HPV, smoking, alcohol use, smokeless tobacco products, and defective systemic immune responses. While HPV has been detected in both precancerous and frank oral cancer lesions, a strong link has been detected between HPV infection and OPSCC (3, 4).

When compared to HPV negative HNSCC, the HPV positive HNSCC patients had better prognosis, improved responses to therapy, and higher survival rate (5-7). The population level incidence of HPV positive cancer has increased from 1988 to 2004 in the United States with a 225% increase in the rate of HPVs-positive OPSCC (from 0.8 per 100,000 to 2.6 per 100,000) (8). There was a concurrent 50% decrease in the rate of HPV-negative OPSCC (from 2.0 per 100,000 to 1.0 per 100,000) (8). It is hypothesized that the changing rate of HPV-positive OPSCC is related to oral HPV exposure secondary to sexual behavior (8). Factors associated with HPV positive oral cancers include history of sexual contact, number of sexual partners, sexual behavior, smoking, and age (5, 7, 9, 10). HPV positive OSCC and OPSCC were more likely to occur at younger ages and were increased in those who had more sex partners and in those who were heavy smokers (5, 7, 10). There are multiple factors that account for differences in reported HPV associated cancer prevalence rates. The prevalence of HPV related OPSCC
varies between studies based on the population, geographic location of the study, the sample size, the quality of specimen, the HPV detection technique, and the classification of head and neck site.

Historically, Syrjanen et al. in 1983 were the first to suggest that HPV infection was involved in the development of OSCC. From 40 OSCC samples, 16 samples demonstrated morphological evidence of HPV infection and half of those sample contained HPV positive nuclei. HPV6, 11, 16, and 18 DNA were then positively detected by in situ hybridization and PCR (11, 12). Subsequent, molecular and epidemiologic studies have provided supporting evidence proving a role for HPV infection in HNSCC and OSCC. Using a generic probe, Gillison et al. assessed 253 samples with a histology confirmed diagnosis of HNSCC and detected HPV DNA in 25% of these samples. The probes detected high risk HPV type 16 in 90% of HPV positive samples while low risk HPV type 11 was identified in one oropharyngeal tumor (5). The overall prevalence of HPV related OPSCC is about 25% (4, 5, 13). The same group conducted a national cross-sectional study and determined that the prevalence of oral HPV infection in the United States citizens, aged 14 to 69 years, was approximately 7% and the number was higher in men than in women (7). The prevalence of HPV infection was higher in OPSCC (35.6%) than OSCC (23.5%) (13).

While molecular evidence demonstrated a mix of HPV types of in OPSCC (4, 13-15), high risk types HPV16 and HPV18 are the most commonly identified types in HPV positive OPSCC. The detection of HPV16 and HPV18 was three times higher in dysplasia and invasive cancer lesions when compared to normal non-hyperplastic tissue control (4). HPV16 has been detected in 90% of HPV related OPSCC (9, 13). Of the HPV16 positive cancers, 50% demonstrated viral integration, 35% demonstrated viral episomes, and 17% demonstrated a mix of integration and episomal types (15). A mix of high risk and low risk HPV types had been detected in hyperplastic oral lesions (16). While the high risk HPVs have consistently been identified in OPSCC, low risk HPVs cause genital tract, oral cavity, and respiratory tract benign
hyperplastic lesions. Although low risk HPVs are regarded as non-oncogenic agents, they have been detected in OPSCC and oral SCC lesions (13). HPV6 has been found in tonsil carcinoma and oral biopsies with histologic evidence of papillomatosis (17-19). HPV6 and HPV11 are the dominant HPV types found in recurrent respiratory papillomatosis; respiratory tract papilloma lesions in children among 1-5 years of age (19). The mucosal and cutaneous papilloma lesions caused by low risk HPVs can be eradicated by surgery or spontaneously reduced by host immune responses. However, the recurrence after treatment is the main problem of these lesions.

HPV associated disease has been detected in the context of HIV. About 14% to 35% of HIV positive patients shed oral HPV (20-22) (Webster-Cyriaque, not published). With the increasing burden of HIV infection worldwide, low risk HPV associated oral warts have increased as well. Oral warts were associated with a greater than 1 log10 decrease in HIV-1 RNA (23). Greenspan et al. retrospectively studied 1,280 HIV patients who received highly active antiretroviral therapy (HARRT) from 1990 to 1999 and detected an increasing in oral warts whereas reducing of candidiasis, hairy leukoplakia and Kaposi’s sarcoma (24). Their results suggested low risk HPVs induced oral warts as a complication in HAART (25). Further this group detected a higher proportion of individuals on HAART with oral warts (23%) than among those not taking HAART (5%) (25). Importantly, recent studies show higher risk of OSCC in HIV-infected patients compared with general population (26-28).
HPV classification and genome organization

Figure 1.1 Genotype of papillomaviruses modified from de Villiers et al, 2013 (29).

Genera

Human HPVs have been divided into five genera; alpha, beta, mu, nu, and gamma, based on DNA sequence analysis (Figure 1.1). Different HPV types are defined by L1 sequences that share less than 90% sequence similarity to the closest related types. The different types of HPV cause different lesions at different sites of infection. The most well studied group is the alpha HPVs since their relationship to cancer is well documented. In general, alpha HPVs infect and cause human mucosal and cutaneous papilloma lesions. While 90% of infected individuals clear HPV infection within 2 years, persistent mucosal infection with
specific alpha high risk HPVs types, such as 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59, may result in the development of carcinoma. High risk HPVs infection is highly associated with cervical cancer in women worldwide, and has been detected in cancers of the penis, vagina, vulva, anal, and of oropharyngeal sites (13, 30, 31).

The other genera; beta, gamma, Mu, and Nu, generally cause cutaneous papilloma. Although the cutaneous papilloma lesions caused by these HPVs genera are unlikely to transform into carcinoma, but the neoplastic formations were found when the infection was occurred in immunosuppressive patients.

**HPV genome organization**

HPVs are small non-enveloped double stranded DNA viruses that are a part of the *Papillomaviridae* family. The HPV particles are approximately 55 nm that encapsulate an approximately 8 Kb genome. The HPV genome can be divided into three major regions, early, late, and long control region (LCR). The early genes E1, E2, E4, E5, E6, and E7 encode nonstructural proteins. Late genes L1 and L2 encode capsid proteins, and the LCR is a non-coding promoter region that regulates viral replication and gene expression. Transcription may be bicistronic, tricistronic, or polycistronic based on splicing of the HPV open reading frames (ORFs). Genome organization and gene functions are similar in each of the HPV types. However, there are differences in the promoter and in polyadenylation sites. The HPV16 genome contains two promoters: p97 and p670, and two polyadenylation sites: early pA (Aₑ) and late pA (Aₗ). Early gene expression is regulated by p97 located in LCR where consensus E2 binding sites, ACC (Nₑ) GGT, interact with cellular transcription factors or the viral E2 to regulate p97 transcription. Promoter p97 control HPV transcription both at basal epithelial cells and at supra-basal differentiated epithelial cells. For late gene expression, there is another promoter, p670, located within the E7 ORF to control HPV L1 and L2 transcription. p670 is active only at supra-basal differentiated cell layers (32).
Different from HPV16, low risk HPVs- HPV6 and HPV11, have three promoters located in the E1, E6, and E7 ORFs (at nucleotides 90, 264, and 674-714, respectively). The HPV6 and HPV11 genomes contain two polyadenylation sites; an early poly(A) site at nucleotide 4371 and a late poly(A) site at nucleotide 7457 (33, 34). Low risk HPVs contain two E5 segments, E5A and E5B. By having two E5 ORFs, low risk HPV is different from high risk HPVs who contain only one E5 segment (Figure 1.2). However, E5B protein is the most to be studied since its similarity to Bovine papillomaviruses (BPVs) and the frequency of naturally transcribed (35).

**Figure 1.2. The genome organization of HPV11 and HPV16** modified from Baker et al, 1996 (33).
The HPV life cycle

**Viral entry and genome maintenance occur at the basal cell layer:**

The HPV life cycle is tightly linked to epithelial cell differentiation. Viruses do not encode their own transcription protein except E1 helicase, therefore, HPV replication requires host cellular DNA synthesis machinery. HPVs firstly infected basal epithelial cell by invading through micro-abrasion or intra-epithelial wound from upper epithelial layer. It is still unclear how HPV successfully use host cell receptors for its own invasion. Most likely that HPVs utilize heparin sulfate proteoglycans for initial attachment with its L1 capsid protein. Integrins α6, β4 and β1 are thought be involved in HPV binding as secondary receptors. Post attachment, there are three potential viral entry modes: clathrin-mediated endocytosis, caveolar endocytosis, and a clathrin- and calveolar-independent pathway. A conformational change of L2 helps the viral particle to bind to a secondary receptor, facilitating viral internalization and viral transport into the cell nucleus. Following the uncoating process, L2 protein forms a complex with the viral DNA, while L1 protein undergoes lysosomal degradation. HPVs DNA replicate in the nucleus. In the basal cell layer, HPV replicates in actively dividing cells and maintains its genome in daughter cells as an episome form at approximately 50-100 copies per cell (36). The active division of basal stem cells enhances viral DNA amplification. At the initial amplification phase, E1 and E2 gene expression is required.

E2 is a transcription factor that binds at multiple ACCN6GGT consensus binding motifs in the LCR to initiate viral replication. There are four E2 binding sites on the LCR thought to be the primary sites for E2 binding at the start site for promoter activation. E2 shares binding sites with other transcription factors such as SP1 and TATA-box binding protein (TBP). E2 acts as both activator and suppressor for HPV replication. Whenever E2 is highly expressed, it competes for binding sites with other transcription factors resulting in repression of E6 and E7 expression and HPV replication. This process may reflect self-regulation to limit levels of viral DNA amplification.
at the basal cell layer facilitating viral escape from immune responses. On the other hand, HPV E2 recruits viral E1 helicase to E1 binding sites on the LCR. E1 expression is low and has weak affinity for its binding site. However, the E1/E2 heterodimer complex has high affinity for E1 binding sites and promotes the accumulation of additional E1 to the E1 binding site. Therefore, E1 requires E2 for targeting to its binding sites on LCR (37). Viral early proteins E6 and E7 are expressed at low levels in basal cells and their role here is for S-phase initiation.

**Viral genome amplification at supra-basal cell layers:**

The early promoter p97 is located in the LCR and controls the expression of E6 and E7. At supra-basal layers, E6 and E7 modulate cell proliferation and promote cell cycle re-entry into S-phase. To activate viral promoters, cell differentiation is required. E6 and E7 prolong cell cycle entry, and delay terminal differentiation to increase the opportunity for viral genome amplification. At supra-basal cell layers both early and late promoters are activated. The differentiation-dependent promoter p670 is located in the E7 open reading frame and its activation increases the expression of replication proteins E1 and E2 as well as other early viral proteins, E4 and E5. High risk E7 stimulates cell cycle progression by binding and inducing the degradation of retinoblastoma protein (pRb) family members (p105, p107, and p130) that act as regulators of cell cycle entry. p105 and p107 are involved with cell cycle entry in the basal cell layer, and p130 is involved with cell cycle re-entry in upper epithelial layers. pRb binds to the E2F proteins, a family of transcription factors that controls S-phase entry and re-entry initiation. Low risk E7 has low binding affinity for p105 and p107 compared to high risk E7, but still binds and degrades p130 and promote the increasing of cell proliferation and viral genome amplification. E7 also binds to cyclin-dependent kinase inhibitors, p21 and p27, allowing for the formation of cdk/cyclinE/p21 or p27/E7 complexes induced S-phase progression. In settings of high p21 or p27 levels, cyclinE/cdk is inactive even with the expression of E7 and cell cycle progression is stalled. On the contrary, low p21 or p27 levels or high E7 proteins expression,
leads to cyclinE/cdk activity, p21 or p27 inactivity, and resultant cell cycle progression. E7 alone may not be able to overcome the block of cell cycle entry. Hence, the important role of E6 protein is to complement E7 function. High risk E6 contains a PDZ-binding motif that is not found in low risk E6. The PDZ binding motif binds with high affinity to PDZ targets which are involved with proteins related to cell proliferation and cell signaling including; PSD-95: a 95 kDa protein involved in signaling, Dlg: the Drosophila discs large protein, and ZO1: the zonula occludens I protein which is involved in maintenance of epithelial polarity. Both high and low risk HPVE6 inactivate cell pro-apoptosis protein p53 but only high risk E6 mediates p53 degradation. High risk E6 forms a trimeric complex composed of E6/p53/ cellular E3 ubiquitin ligase (E6AP) that induces the ubiquitination of p53 and subsequent proteasome degradation. E6 associates with other pro-apoptotic proteins, Bak and Bax to inhibit cell apoptosis. Moreover, high risk E6 up-regulates telomerase activity through the activation of telomerase reverse transcriptase (TERT) and maintains telomere integrity during cell division.

E4 and E5 proteins also contribute genome amplification. E4 is highly accumulated during DNA synthesis and is thought to cause G2 phase cell cycle arrest and to play a role in viral egress and transmission. E5 has been shown to support genome amplification through EGFR stabilization, enhanced EGFR down-stream signaling, and interference with cell apoptosis (38). Viruses do not express viral epitope in these supra-basal epithelial cell layer thereby help for HPVs to escape from host immune surveillance and allow for prolong viral episomal staying in epithelial cells.

**Viral assembly and release:**

At the uppermost epithelial cell layers, HPVs encode structural proteins L1 and L2 for viral genome packaging and particle assembly. Viral capsid protein synthesis is dependent on mRNA splicing to initiate use of both the late promoter and late polyadenylation site. The E1^E4, E5 message associated genome amplification is switched to E1^E4, L1 messages for genome
packaging. L2 protein accumulates at nuclear promyelocytic leukaemia (PML) bodies prior to L1 recruitment to nucleus. HPVs contain 360 copies of L1 protein and 12 copies of L2 protein that form an icosahedral capsid containing 72 capsomeres in the nucleus. E4 protein is highly expressed in upper epithelial layers and is thought to disrupt the keratin network to compromise the assembly of the cornified envelope. This E4 associated activity is important for virus release and further infection. The accumulation of the immunogenic late viral proteins, L1 and L2, to complete viral particles at the uppermost layer is another factor that helps virus to escape from host immune responses. Newly formed viral particles are shed from the uppermost layer of epithelial where immune cells are generally not present.

Persistent HPV infection is key to HPV-associated squamous cell carcinoma formation. Almost of HPV infections are detected and eliminated by the host immune system. HPV infected tissues that remain may form papilloma lesions that are self-eliminated or can be surgically removed. A small percentage of prolonged HPV infections with episomal viruses and/or integrated viral DNA may induce chromosomal abnormalities and abrogate normal DNA damage responses. In case that these viral induced host DNA abnormalities are not be able to diminish by host immune responses, HPV infection may result in cancer formation.

**HPV E5: a small protein plays important role on HPV related head and neck tumor**

Most HPVs studies are focused on the role of E6 and E7 in carcinogenesis. However, the role of HPVE5 on cancer formation and its mechanisms to support E6 and E7 function remains unclear. Low risk HPVE5 function is less well defined compared to high risk HPVE5 as there have not been abundant studies to determine its role.

HPVE5 is a small hydrophobic protein composed of three trans-membrane domains and localized mainly at the endoplasmic reticulum and Golgi apparatus (35, 39). HPV16E5 has been found to also localized at plasma membrane in HaCaT cells (40). Although the high risk and low risk HPVE5 share small number of amino acid sequences (Figure1.3), they maintain similar
functional domains with similar activities (38). HPV16E5 protein is about 83 amino acids long and HPV6E5 is about 91 amino acids long. Both HPV16E5 and HPV6E5 share structural similarities with type I bovine papillomavirus (BPV1).

Figure 1.3. Cartoon showing the differences between high and low risk E5. Amino acid sequences are aligned. Conserved amino acids between high and low risk E5 are about 15% and are highlighted in red (modified from Conrad and Schlegel, 1993) (35).

In humans, the HPVE5 protein has been detected in all levels of cervical neoplastic lesions, i.e. in lower third of low-grade squamous cell intraepithelial lesions (LSILs), throughout the epithelium of high-grade squamous cell intraepithelial lesions (HSILs), throughout cervical intraepithelial neoplasia (CIN) I, II, III, and in HPV episome containing invasive cervical carcinoma (41, 42). The E5 open reading frame is most likely deleted upon viral integration into host DNA, however, recent study by Schegel et al. detected expression of HPV16E5 in Caski and HPV16 transfected COS cells (42, 43). The expression of HPV16E5 in clinical cervical specimens and cervical cancer cell lines suggests that HPV16E5 may potentially be expressed
post integration and/or that episomal HPVs may contribute to cervical and also HNSCC cancer formation.

**High risk and low risk HPVE5 and cell transformation**

: HPVE5 proteins have been classified into four groups; E5α, E5β, E5γ, and E5δ, by their chemical characteristics and their phylogenetic relationships associated with E5 related transformation. All E5 groups share the highly hydrophobic trans-membrane region containing Ile+Leu+Val amino acids that are related to E5’s effects on host cell proliferation, apoptosis and resistance to immune responses. E5α is the group that is associated with malignancy and HPV16 and HPV18 E5s are categorized into this group. E5β is related to warts while E5γ and E5δ is related to benign tumors (44). In contrast to the high transformation activity of BPV-1 E5, both HPV16E5 and HPV6E5 weakly transform rodent and human cells (45, 46). HPV6E5 induced anchorage-independent growth of NIH 3T3 and C127 cells (47) while HPV16E5 was shown to transform human, mouse, and murine fibroblasts and to increase keratinocyte transformation activity when co-expressed with HPVE7 and EGF (45, 46, 48, 49). The collaboration with E6 and E7 resulted in higher risk for cell transformation in high risk HPVE5 containing cells. Moreover, HPVE5 induced koilocyte formation; a morphological marker of HPV infection identified by large pyknotic nuclei with prominent nucleoli surrounded by clear cytoplasm (50). The koilocyte formation is thought to promote nuclear membrane fusion.

**HPVE5 function on cell proliferation and differentiation**

: High risk and low risk HPV E5 proteins share similar cell proliferation induction ability through EGFR signal transduction. HPV16E5 and HPV6E5 are membrane associated-proteins that bind to vacuolar ATPase (35). It was initially believed that by binding to ATPase, HPVE5 impaired endosomal acidification and reduced EGFR degradation, therefore, increasing EGFR cell surface recycling (51-53). However, others suggested that HPVE5 inhibited EGFR trafficking to late endosome (54) and did not directly disrupt V-ATPase function (55). In the
presence of EGF, both high risk and low risk HPVE5 up-regulate phosphorylated EGFR levels and phosphorylated MAPK signaling proteins down-stream of EGF receptor (38, 56, 57). HPV16E5 has been shown to increase phosphorylated EGFR in raft culture (52). HPV16E5 mediated cell transformation occurs through EGFR signaling pathway via Ras-Raf-MAP kinase and PI3-Akt pathways. Collaboration between HPV16E5 and EGFR promoted cell cycle progression and induced S-phase entry of both human keratinocytes and fibroblasts (45, 48). HPV16E5 and HPV31E5 transfected keratinocyte cells showed enhanced EGFR activity and enhanced expression of MAPK protein and Erk1/2 (58, 59). E5 expressing cells demonstrated higher levels of p-Akt and VEGF receptor in HaCaT and C33A cervical cancer cell lines (58). However, there is still controversy regarding the necessity of EGFR for HPVE5 function. A study from Crusius et al. demonstrated that HPV16E5 was able to modulate Erk1/2 and p-p38 expression in human keratinocytes in an EGFR-independent manner under conditions of cell stress (57). In addition, the presence or absence of EGF in the cells that were not expressing E5 did not contribute to the hyperactive growth of keratinocyte cell (60). HPV16E5 has been demonstrated to activate VEGFR (58) and KGFR/FGFR2b via the modulation of MEK1/2, Erk1/2 and PI3K pathways (61). In summary, HPVE5 activation of epithelial receptors allows for prolonged cell proliferation and hyper proliferation.

The complete mechanism of HPVE5 induced cell transformation is still unclear. In a transgenic mouse model, HPV16E5 enhanced severe neoplastic head and neck carcinomas compared to control mice. The carcinomas were more severe in E5E6E7 transgenic mice than mice that expressed a single HPV oncoprotein or one transgene (62). Higher levels of Erk1/2 were shown in E5 transgenic mice (62). HPVE5 to EGFR interactions are believed to be the major cause of HPVE5 related carcinoma formation. In vivo data indicated a role for E5 in cell transformation and a role for potential synergism with E6 and E7 in cancer development.

There is some evidence supporting a role for high risk HPVE5 in cell cycle progression and viral particle production (59, 60, 63). It has been shown that keratinocytes transfected with a
mutated HPV31E5 demonstrated less HPV late gene production, lower levels of cell cycle related proteins, less cyclin A and cyclin B, and less colony formation (59, 64). Keratinocytes harboring a mutated HPV16E5 showed less viral DNA synthesis and less viral particle formation in differentiated raft cultures, suggesting that E5 is critical to productive HPV infection (60).

**HPV E5 and immune responses**

There are few studies on HPVE5 and human immune responses. In HaCaT cell lines, HPVE5 suppressed the expression of HLA class I and HLA class II on cell surfaces (65-67). HPV16E5 binds and arrests HLA class I at the Golgi apparatus through the direct interaction of E5 with the heavy chain of HLA class I complex (68). The suppressive effect of HPVE5 is selective for HLA-A and HLA-B, whose functions have no effect on NK cell. Therefore, HPVE5 suppression of HLA class I does not trigger NK cells. Further, HPV16E5 suppresses HLA class II by inhibiting the maturation of HLA class II via interrupting the breakdown of the invariant chain (67).

E5’s impact on IFN responses may be important for tumor progression. Interestingly, HPV16E5 induced the expression of type I IFN through the IRF-1 activated pathway in HaCaT and C33A cells (56). However, the exact mechanism was not determined. Studies from Stanley group detected increased integrated HPV in W12 cell lines when IFN was introduced into the cells (37, 69). Long-term interferon treatment of HPV31-positive cells was associated with reduced HPV episome levels but did not decrease HPV integrated copy number (70). Recently, Lace et al. treated human foreskin keratinocyte cells with IFN-g demonstrating an increase in integration by ≥100 fold (71). The potential exists that E5 may be critical to these processes, this it is important to understand the role of HPVE5 on innate immune responses and their roles in tumor progression since this may be the critical step of cancer formation in HPV infected cells. This is addressed in chapter 2 of this thesis.
HPVs modulate cellular immune responses

HPVs infect by obtaining access through micro-abrasions of epithelial surfaces. While immune responses should successfully eliminate about 80-90% of HPV infections and resolve the HPV infected lesions, the prolonged retention of HPV in epithelial cells may result in the development of intraepithelial lesions or transformation to invasive squamous cell carcinoma (72). The cell-differentiation dependent HPV life cycle impacts host immune responses to HPV infection. The professional antigen presenting cells, dendritic cell, macrophage and Langerhans cells that are present in supra-basal cells are important to HPV detection, antigen processing, and presentation to naïve T cells. Post presentation, effector T cells may migrate to the infected site to eliminate HPV infected cells. CD4+ and CD8+ T cells are critical to decreasing HPV infection in CIN and invasive squamous cell carcinoma lesions. Histological studies of regressing genital warts demonstrate significant migration of CD4+ T cells, CD8+ T cells, and macrophages (73). Study in canine oral papillomavirus infection (COPV) demonstrated a peak in CD4+ responses to COPV E2 and E6 concurrent with the regression of COPV warts. The L1 neutralizing antibody emerged afterwards and was remained for months before a slow decline (74). CD8+ T cells are critical to HPV clearance in human CIN1 lesions since CD8+ cells were detected at high levels by immunohistochemistry in CIN1 lesions and correlated with lesion regressions (75). Low risk HPVs clearance is shorter, taking only 4-8 months compared to 8-16 months of high risk HPVs infection. Cellular responses to HPV infection have been well described. The persistent infection of high risk HPVs may be related to viral oncogene interference with type I antiviral IFN responses. The appendix of this thesis demonstrated the local and systemic immune responses to oral HPV in HIV infected patients.
Intracellular viral recognition

Both innate and adaptive immune responses are required for viral clearance. Innate immunity serves as the first line of defense to elucidate the microbial infections. Invaded by pathogens and their associated antigens, the innate immune sensors initiate the clearance processes. Host immune defenses against viral infections are initiated by the recognition of viral genomic RNA or DNA or double-stranded RNA (dsRNA) as pathogen-associated molecular patterns (PAMPs) by host pattern-recognition receptors (PRRs). Four main PRRs function as primary sensors to detect PAMPs and include Toll-like receptors (TLRs), DExD/H box RNA helicase receptors, Nod-like receptors, and C-type lectins (76-79). Viral DNA can trigger all TLRs, RNA helicase receptors and DNA sensors. Intracellular DNA sensors are distinct and can be discriminated from TLRs and RNA helicase by their ability to initiate IFN response pathways. Viral DNA motifs trigger PRRs and initiate signaling cascades to activate IFN-regulatory factors (IRFs) and nuclear factor kappa B (NF-κB). The accumulation of IRFs and NF-κB in the nucleus activates type I IFN expression and further stimulate the expression of IFN-stimulated genes and pro-inflammatory cytokines.

Innate immune responses, MAPK, and viral infection

Type I IFNs, IFNα and IFNβ are the first line of defense for virus infection and bridge innate and adaptive immune responses. In an infected cell, DNA sensors transmit signals to IRF3, IRF7, and to NF-κB to activate type I IFNs that may affect adjacent HPV infected cells. Once type I IFN receptors bind to IFNα and IFNβ, the sequential cascades begin from the phosphorylation of Jak1 and Tyk2 to activate the phosphorylation and dimerization of STAT1 and STAT2. The activated STAT1/STAT2 heterodimers translocate into the nucleus where they bind to IRF9 forming an ISGF-3 complex that stimulates the interferon regulatory elements (ISREs). ISREs activation contributes to the expression of IFN stimulated genes (ISGs). Similar to type I IFNs, type II IFNs induce the expression of many ISGs that inhibit viral replication and destroy HPV infected cells. IFNγ binds to type II IFN receptors and activates signal transduction.
through Jak1/Jak2 and Stat1/Stat1 homodimers. The homodimer STAT1/STAT1 translocates into the nucleus and regulates the expression of the GAS element, an activator of ISGs expression.

The activation of MAPK pathway stimulates the expression of type I IFNs and pro-inflammatory cytokines such as IL-6, IL1β, and TNFα (80, 81). Among others, p38 MAPK has been shown to regulate ISRE and GAS elements that further induce type I IFN responses in hematopoietic cell lines (82). In HeLa cells, p38 acted as a STAT1 serine kinase and stimulated IFN promoter (82). p38 MAPK and Stat1 act as regulators of pro-inflammatory cytokines and of IFNβ induction in epidermal keratinocytes (83).

Molecules important to the intracellular detection of viral infection modulate both MAPK and interferon responses. There are multiple pathways that demonstrate a link between MAPK and innate immune responses. For example, TLR3 is an intracellular TLR that recognizes double-stranded RNA (dsRNA) and poly inosinic acid-cytidylic acid (poly I:C); a synthetic analog of viral RNA that has been widely used in molecular studies of IFN response. TLR3 activates TRIF, TRAF6, IKKβ, and MAPKs to subsequently stimulate pro-inflammatory cytokines and type I IFN expression. The cytosolic DExD/H box RNA helicases act as receptors for viral RNA and include the retinoic acid inducible gene I (RIG-I) and melanoma differentiation associated gene 5 (MDA5). RIG-I is activated by short blunt end dsRNA with a 5’ triphosphate molecule while MDA5 recognizes long dsRNA. RIG-I and MDA5 broadly are able to detect viral nucleic acid; negative-strand RNA viral infection, single stranded-plus strand RNA viral infection and actively transcribed DNA viruses. RIG-I and MDA5 mediated type I IFN responses are IPS-1 dependent. IPS-1 is a mitochondria and peroxisome associated protein that contains CARD like structure and functions as adaptor for RIG-I/MDA5 (84). Activated IPS-1 then interacts with STING that further facilitates the activation of IRF3, NF-κB, MAPK, TBK1 and IKKi pathways. STING is an important cytosolic linker for viral RNA and DNA receptors and type I IFNs responses (76, 79, 85). STING up-regulates TBK1 and IKKi signaling whose down-stream activation is to stimulate
the nuclear translocation of IRF3 and NF-κB and the expression of type I IFNs, ISGs, and pro-inflammatory cytokines to counteract viral infection. The potential that recognition of HPV viral RNA results in the up-regulation of the type I IFN response is demonstrated in chapter two of this dissertation. In chapter 2 of this thesis, the potential for both HPVE5 mRNA and protein to activate the IFN response is assessed.

HPV oncoproteins and innate immune responses

HPVs successfully escape host immune responses by many mechanisms. First, HPV replication takes place in supra-basal cell layers far from typical sites of immune cell activity. HPV infection does not result in lytic infection and does not have a blood borne phase, hence HPV infection activates only small number of dendritic cells. HPV positive cells have also been shown to present low level of cell surface MHC class I and MHC class II molecules. High risk HPVE5 disrupts exocytotic and endocytotic trafficking including the transportation of MHC class I (65, 66). Further, the inhibition of breaking down of invariant chaperone (Ii) by high risk HPVE5 blocks the loading of viral peptide onto immature MHC class II molecules and resulting in diminished of surface MHC class II dimer expression (67).

High risk HPVE6 and E7 interfere with IFN responses. Abrogated IRF1 responses were detected in both high risk and low risk HPV infected cells (86, 87). Micro-array analysis identified three groups of genes that were down-regulated in HPV31 infected cells including genes that regulate cell growth, keratinocyte specific genes, and genes associated with IFN stimulation (88). Micro-array analysis of HPV16E6 expressing cells altered IFN-responsive genes, NF-κB stimulated genes, and cell cycle regulatory genes (89). Among the down-regulated genes, STAT1 was a target for both E6 and E7. STAT1 suppression is important for viral genome replication. Restoration of STAT1 using recombinant retroviruses impaired HPV genome amplification of in a cell differentiation dependent manner (90). E7 binds to p48/IRF9 and prevents the ISGF-3 complex translocation into nucleus, thereby inhibiting the activation of ISRE (91, 92). E7 binds to the c-terminal domain of IRF1 inhibiting IRF1 activated IFNβ
production by recruiting histone deacetylase to IFNβ promoter (87). HPV18 E7 was shown to down-regulate IRF1 target genes such as TAP1 and MCP1 (86).

E6 also targets and suppresses IFN induction pathways. HPV16 E6 binds the carboxyl domain of IRF3 and deregulates IRF3 transactivation, preventing the transcription of IFNβ (93). HPV18E6 also decreased IFNα expression by binding to Tyk2 and inhibiting the phosphorylation of Tyk2, STAT1, and STAT2, thereby inhibiting Jak/Stat formation and IFNα inducing pathways (94). High risk and low risk HPVE6 differed in their abilities to abrogate IRF3 and Jak/Stat pathway activation. In sum, E5, E6, and E7 alter the expression of HLA class I and II, IFNs, ISGs, and cytokine responses to avoid viral clearance by host immune responses. The effects of E6 and E7 on immune responses in oral epithelial cells are assessed utilizing an E5 deletion mutant in Chapter 2 of this thesis. While E6 and E7 effects on innate immunity have been well studied, the role of E5 has been understudied.

Muto el al. determined that E5 to induced IFNβ expression in HaCaT and C33A cell lines (56). This seems to contradict to the suppressive effects of E6 and E7. HPVE5 mediated IFNβ expression was regulated by IRF1 in a dose dependent manner, and was unrelated to IRF3 and IRF7 in HaCaT cells. HPV16 E5 also significantly induced the activation of GAS and NF-κB promoters at 24 hours post-transfection (56), thus High risk HPVE5 may affect both IFN responses and pro-inflammatory cytokine induction in HaCaT cells. The ability of E5 to modulate interferon responses in oral epithelial monolayers and rafts is assessed in chapters 2 and 3, respectively, in this thesis.

Contributions to the field of study

As reviewed above, high risk HPV infection contributes to oropharyngeal malignancy while low risk HPV is highly associated with oral wart formation. HPV is a growing problem, particularly in the context of HIV/AIDS. We seek to understand how HPV infection modulates oral immunity. The high risk HPV type 16, is associated with 90% of HPV-associated oropharyngeal malignancy (13) while low risk HPVs, such as type 6 and 11 have are associated
with the increasing rate of oral warts in HIV-infected individuals on HAART treatment (25). The role of high risk HPVE5 on signal transduction and its role on cell apoptosis and MHC suppression have been examined in previous studies (58, 65). There has only been one previous study in cervical cells assessing HPV16E5 and immune responses and mechanisms were not explored. The impact of high risk and low risk HPVE5 on oral epithelial cell host responses have not been previously studied.

We hypothesized that 1) high risk but not low risk HPV E5 modulate MAPK pathways to promote type I IFN responses in oral keratinocytes (Chapter 2), and that 2) high risk HPVE5 delays terminal differentiation to promote cell proliferation and type I IFN responses in a cell differentiation dependent manner (Chapter 3). Furthermore, we investigated the potential role of oral HPV to local oral immune activation in the context of HIV infection (Appendix) (Figure 1.4).

In this thesis, we determined the role of HPV16E5 and HPV6E5 on type I IFN responses. We investigated the intracellular signaling pathways utilized by HPV16E5 to modulate innate immune responses. These experiments were conducted under three specific aims (Figure 1.4).

**AIM 1:** To determine the contribution of HPVE5 to the activation of type I IFN responses in oral epithelial cells.

1.1 Determine the role of HPVE5 on signaling pathways modulation as a potential mechanism to activate type I IFN responses in oral epithelial keratinocytes.

1.2 To determine whether high and low risk HPVE5 gene products differentially activate type I IFN responses in oral epithelial keratinocytes.

1.3 To determine the role of EGF on HPVE5 induced type I IFN responses

**AIM 2:** To determine epithelial host factors critical to HPVE5 mediated activities.

2.1 To determine the role of HPVE5 on cell differentiation and on differentiation associated type I IFN responses
AIM 3: To access oral HPV and oral markers of inflammation/immune activation in patients with HIV infection.

3.1 Determine prevalence of oral HPV infection in the UNC HIV dental population

3.2 Determine SLPI, sCD14, sCD163, IL-6, IP-10, and IgA levels in HPV positive and negative individuals.

Figure 1.4 Oral HPVE5 study framework
REFERENCES


CHAPTER 2: HIGH AND LOW RISK HPV E5 DIFFERENTIALLY MODULATE THE TYPE 1 ANTIVIRAL IMMUNE RESPONSE IN ORAL KERATINOCYTES

Abstract

The high (HR) and low-risk (LR) Human Papillomaviruses (HPVs) cause benign and malignant lesions of the oropharynx. Cell proliferation and activation of intracellular immune responses are critical to transformation of oral keratinocytes and cancer development. The hydrophobic HPV E5 proteins are associated with cell growth deregulation, survival and differentiation through the modulation of growth factor receptors. To better elucidate HR-HPVE5 and LR-HPVE5 regulated molecular events central to disease development in oral epithelia, proliferative signals and type I interferon responses were assessed. Quantitative reverse transcriptase–PCR, biochemical approaches and immunofluorescence were applied to oral keratinocytes (OKF6 cells) transfected with pCMV plasmid vectors containing either whole HPV16 genomes with E5 (HR-HPV16WT), HR-HPV16E5 genome alone with HA-tagged (pCMV16E5HA), deleted E5 (HPV16ΔE5), transcribed but un-translated HPV16E5 (HPV16E5stop) (in the context of the whole genome or over expressed), and LR-HPV6E5 genome alone with HA-tagged (pCMV6E5HA). HR-pCMV16E5HA demonstrated consistent phosphorylation of MAPK family members, p38 MAPK, MEK1/2 and Erk1/2 proteins. Phosphorylation of MAPKs was increased with HPV16WT and pCMV16E5HA but not with pCMV6E5HA over-expression. This was confirmed by pharmacologic perturbation. HR-HPV16E5 mRNA maintained Erk1/2 activation, implying a role for E5 transcripts in MAPK modulation. HR-pCMV16E5HA suppressed phospho-AKT but phospho-JNK levels were not different between HR-pCMV16E5HA and LR-
pCMV6E5HA. HR-pCMV16E5HA protein induced the Type I interferon responses (ISG54, STAT3, 2’5’OAS) at promoter (P = 0.028), transcript and protein levels while LR-pCMV6E5HA IFN responses were comparable to vector control. Pharmacologic inhibition demonstrated that E5 mediated type I interferon responses were p38 dependent. HR-pCMV16E5HA activation of Erk1/2 was EGF independent while induction of ISG54 transcripts was EGF dependent. HR-pCMV16E5HA inhibited EGF dependent splicing of E6 transcripts while HPV16ΔE5 and HPV16E5stop did not. In summary, through modulation of proliferative signals and type I interferon responses, HR-HPV16E5 may be an important facilitator of oncogenic progression in the oropharynx while LR-HPV6E5 is not.

Introduction

Over 170 Human Papillomavirus (HPV) types have been detected in cutaneous and mucosal epithelium (1, 2). Persistent oral HPV infection may manifest as benign hyper-proliferative lesions or may progress to malignancy (3, 4). Of the five human papillomavirus genera, alpha papillomaviruses demonstrate the most significant association with malignancy (2). Alpha HPVs are subdivided into the high-risk (HR) and low-risk (LR) types based on their oncogenic association. The HR-types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59 are considered cancer causing, while the LR-types 6, 11, and others are detected in benign warts and respiratory papillomatosis lesions (1, 4).

Head and neck squamous cell carcinomas (HNSCCs) are the sixth most prevalent cancer worldwide with an estimated 405,000 new cases and 200,000 deaths each year (5, 6). In the United States, the population-level incidence of HPV-negative HNSCCs declined by 50% due to public health efforts around smoking cessation, while HPV-positive oral squamous cell carcinomas (OCSSs) increased by 225% from 1988 to 2004 (7). HR-HPVs are the causative agents for oral cavity and oropharyngeal squamous cell carcinomas (8-10). A recent systematic review determined that HPV was associated with OCSSs (35.6%) and that HPV16 accounting
for the majority of HPV-positive OSCCs (86.7%). Interestingly, HPV6, designated a low risk virus, was detected in 2.5% of OSCCs (9). LR HPV associated oral warts have been detected during immune suppression and were detected at increased incidence among HIV patients on highly active antiretroviral therapy (HAART) (11). The increase in HPV-associated oral lesions highlights the importance of understanding oral HPV pathogenesis.

HR-HPVs encode three major oncoproteins: E5, E6, and E7. HPV oncoproteins induce chromosomal abnormality, hyper-proliferation and interrupt normal cell growth, which eventually causing keratinocyte immortalization and transformation (12-14). In combination with HPV16E6 or E7, HPV16E5 induced cervical carcinogenesis in transgenic mice (15). In contrast, LR-HPVs E5, E6, and E7 did not immortalize human keratinocytes. The binding affinity of HR-HPVE7 for retinoblastoma tumor suppressor protein (pRb) and of HR-HPVE6 for p53 binding is significantly higher than that of LR-HPVE7 and E6 counterparts. While HR-HPVE7 and E6 mediated loss of cell cycle control are well described as a critical step in cell transformation, the activity of E5 proteins in human keratinocytes is still unclear.

HPV E5 is a small hydrophobic trans-membrane protein predominately localized at the endoplasmic reticulum, Golgi apparatus and plasma membrane (16-18). While HR- and LR-HPVE5 share limited amino acid homology (Figure 2.1), they maintain similar functional domains that are associated with disruption of normal epithelial cell function by V-ATPase binding, mediating endosomal acidification, and down-regulating HLA class I (19-23). Both HR-HPV16E5 and LR-HPV6E5 induce koilocyte formation, a morphological marker of HPV infection (24). In the presence of EGF, both HR-HPV16E5 and LR-HPV6E5 transformed rodent fibroblast cell lines and primary cells in soft agar and activated both the EGF and VEGF receptors by up-regulating the phosphorylation of cell signaling molecules (22, 25, 26). HPV16E5 transfected cells were more mitogenically active comparing to HPV6E5 (13, 27). Cell growth induction and cell apoptosis disruption functions of HR-HPVE5 were strongly related to cell transformation and cancer formation (22). HPV16E5 elevated EGFR at the cell surface, and increased its recycling
HPV16 positive tumors with high E5 expression levels had significantly higher EGFR levels \((p=0.03)\) and E5 and EGFR expression were determined to be the strongest predictors of recurrence-free survival \((p<0.01)\) (28).

Another biological role of HR E5 proteins is modulation of MAPK pathways. Bovine Papillomavirus 1(BPV) E5 expressing fibroblasts increased phosphorylated-p38 MAPK \((p-p38)\) levels (29). HPV16E5 increased phosphorylated p38 \((p-p38)\) and phosphorylated-Erk1/2 \((p-Erk1/2)\), the active forms of these kinases, in HaCaT cells under EGFR independent conditions (25). Enhanced p-Erk1/2 and p-PI3K/Akt were observed in HPV16E5 transfected HaCaT, 293T, and C33A cells under EGF dependent conditions (26).

Another biological role of HR-HPVE5 proteins is modulation of the IFN responses. (30). HPV16E5 stimulated IFN-\(\beta\) and double-stranded RNA-dependent protein kinase (PKR) expression through IRF1 (30). In contrast to the role of E5, HR-HPV16 and HPV31 infected cells showed reduced interferon responsive and reduced STAT1 genes expression (31, 32). In order to escape from innate immune surveillance, HPV16E6 and HPV16E7 suppressed STAT1 expression and down-regulated IRF7 and IRF3 (33, 34). HPV E5 is thought to counteract the IFN suppressive activities of E6 and E7 (33, 34). However, the mechanisms of E5 modulated IFN responses are still unclear.

We postulated that HPVE5 modulated type I IFN responses through interaction with MAPK pathways. P-p38 MAPK has been previously shown to act as an activator of interferon stimulated regulator element (ISRE) and IFN-gamma activated site (GAS) element in hematopoietic progenitor cells (35). In epidermal keratinocytes, p38 was not responsible for dsRNA mediated IFN\(\beta\) responses (36) but has been shown to be important for HPV amplification at differentiation stage of keratinocytes (37).

HR-HPVE5 and LR-HPVE5 have not been assessed in the context of oral epithelial cells nor has the impact of LR-HPVE5 on type I IFN stimulation. In the studies presented here, we sought to determine the role of HR- and LR- HPVE5 in the modulation of signal transduction
and type I IFN responses in oral epithelial cells. Proliferative signaling pathways were differentially activated in HPV16E5 expressing cells, in cells expressing E5 deleted HPV16 genomes, and in HPV6E5 expressing oral epithelial cells. Interestingly, only HPV16E5 exhibited enhanced Type 1 antiviral responses.

Materials and Methods

Cell culture. The TERT-immortalized oral keratinocyte cells, OKF6 (obtained from Dr. James Rheinwald) (38), were grown in Keratinocyte serum free media (Gibco) supplemented with 0.2 ng/ml EGF, 25 mg/ml BPE, 10% penicillin-streptomycin and 0.4 mM CaCl$_2$. OKF6 cells were sub-cultured every 3-4 days.

HPVE5 DNA constructs and transfection. The full length HPV16 genome was PCR amplified from oral cancer biopsy DNA using a Roche Expand Long Template PCR System and the primers HPV16BamHIF (5’-CCCGGATCC CCATGTACCAATGTTGCA-3’) and HPV16BamHIR (5’-CCCGGATCC TTTGCCCCAGTGTTCC-3’). The 7.9 kb PCR fragment was TA-cloned into the pCMV vector (HPV16WT). Vector control plasmids containing Haemagglutinin (HA) were designated pCMVHA. pCMVHA was used as the vector for the E5 overexpression plasmids described below with the Haemagglutinin (HA) epitope at the N- or C- terminus. The following E5 constructs were generated using Taq polymerase, listed primers and standard PCR conditions (Figure 2.2A): 1) HPV16WT whole genome 2) HPV16 genome with deletion of the E5 ORF using specific primer for E5 deletion (pHPV16ΔE5): 5’-TTACAGTGTCTACTGGATTATGCAGATTGAAATTTGTTTATTACTAAAATGCG-3’ and 5’-CGCATGTTAAGTAATACAGGATGATTTTA CAGTTTATTCTCGAGTCTATAGACA-3’ (Figure 2.2A and 2.2C), 3) N-terminal HA- tagged HPV16E5 overexpression construct (pCMV16E5): 5’-GCAGGAAGCTTCCATGACATGTTTTGACTGGTTTTA TGTCTATATGAAATTTGTTTATTACTAAAATGCG-3’ and 5’-GCGATCGTGTACCCATA CGATGTCCAGATTACGCTATAGAAATCTTGATACTGC-3’ and 5’-GCGCTCGAGTGTACCCATA TGTAATTAAAAGCGTGAGTTACGCTATAGACAAATCTTGATACTGC-3’, 4) and 5) transcribed but not translated HPV16E5 overexpressed (pCMV16E5top) or in the context of the whole genome (pHPV16E5top) 5’-
CTATGACATAGCTTTAAACTGCATCCA-3’ and 5’-TGGTGCAGTTAAAGCTATGTCATA
TAG-3’ (Figure 2A and 2B), 6) HPV6 E5 with HA-tagged (pCMV6E5): 5’-GCGAAGCTT
ACCATGTACCACGATGTTCCAGATTACGCTGGGAAATCGTACTTTTGTATGA-3’ and 5’-
GTATAACAAAGCATAACTTTACCAA-3’, and 7) pCMV containing IRF7 ORF (pIRF7) (not
shown). The expression of E5 was detected by immunoblotting of the HA-tag and by real time
reverse transcription-PCR (real time RT-PCR). Each set of primers carried HindIII and Xhol
sites to enable cloning into restriction endonuclease sites in pCDNA3 using T4 DNA ligase
(Bethesda Research Laboratories). Plasmids were cloned and selected in DH5α Escherichia
coli (Invitrogen) following manufacturer’s instructions. Constructs were sequenced for
confirmation and the resulting amino acid sequence matched previously published results
(Figure 2.1).

OKF6 cells were grown to 70% confluence and transiently transfected with pHV16,
pCMVHA, pIRF7, and different E5 constructs using Fugene-6 (Roche). pRFP was co-
transfected to determine transfection efficiency. Cells were allowed to grow for 48 hours prior to
harvesting for protein and RNA.

RNA isolation and real-time reverse transcriptase-PCR analysis. Total RNA extracts were
isolated with Trizol (Life Technologies) and 1 μg of RNA was reverse transcribed into cDNA with
SuperScript III (Invitrogen) and oligo (dNTP) priming activation at 50°C for 60 minutes and
inactivation at 70°C for 15 minutes. Real-time RT-PCR was performed in a LightCycler
instrument using the cDNA master SYBR Green I dye (Roche Molecular Biochemicals).

Detection of ISG expression. Real time RT-PCR for type I interferon stimulated gene expression
was performed with specific primers for ISG54: 5’-TTCACCTCTGGACTGGCAATAGCA-3’ and
5’-AAGCTTCAGAGCCAGGGACTTT-3’; and 2’5’OAS 5’-GTTGGTGTTGGCATCTTTCTGG
CAA-3’ and 5’-TTGGGTTCAGCTCCATCCTGA-3’. GAPDH was used as an internal control
under the following PCR condition: initial denaturation incubation at 95°C for 10 minutes
followed by 50 cycles of alternating 95°C incubation for 10 seconds, annealing at 50°C for 10
seconds and extension at 72°C for 30 seconds. cDNA amplification was performed in triplicate. ISGs and 2’5’OAS gene expressions were compared to empty vector pCMVHA before normalized to GAPDH (2-ΔΔCt).

Detection of E5 variants. E5, E2 and E1^E4 mRNA expression in transfected cells was measured using real-time RT-PCR with the following primers: HPV16E5 5’-CATCCACAACATTACTGGCGTGCT-3’ and 5’GCAGAGGCTGCTGTTATCCACAAT-3’, HPV6E5 5’-GGAAGTGGTACCTGTCAAATAGCTG-3’ and 5’-GGGTTGTTAATAGCAGCCACA-3’; E2; 5’-CGACTATCCAGCGACCAAG-3’ and 5’-TGAGTCTCTGTGCAACAATTAGTG-3’, and E1^E4; 5’-CCCATCTGTTCAGTACAAACC-3’ and 5’-GGCCAATGTCTCGCTAATA A-3’. mRNA expression was normalized to GAPDH (Figure 2.2D).

Detection of E6 splice variants. The cDNA from pHPV16WT, pHPV16E5, pHPV16ΔE5 and control vector expressing cells was amplified using HPV16E6F primer: 5’-TGCAATGTTTCGGGACCCACAGG-3’ and HPV16E7R primer: 5’-CCAGCTGGACCATCTATTTCA-3’ under the following PCR condition: 40 cycle amplification at 94°C for 2 min followed by 55°C for 30 second and 72°C for 30 second. The PCR products were separated on 0.7% agarose gel or subjected to quantitative real time RT-PCR using HPV16E6 spliced primer 5’-CGTCGACGTGAGGTTAAC-3’ and HPV16E6E7 primer 5’-GTGTATTAACTGGCAAAAGCCACTG-3’.

Phosphorylated p38 inhibitor and Cox2 inhibitor treatment. At 48 hours post-transfection, culture media was changed to non-EGF KSF media and OKF6 cells were treated with 5 μM Cox2 inhibitor (Celecoxib; Pfizer) for further 48 hours or with 10 μM SB202190 (Sigma Aldrich) for 30 minutes before harvesting for protein and RNA.

Protein isolation and Western Blotting. Cells were lysed and proteins were isolated with RIPA buffer. Protein concentrations were measured using Biorad protein assay kit (Biorad). Protein lysates were separated by SDS-PAGE on 4-12% agarose gel (Life Technologies) and transferred to nitrocellulose membrane. Blots were incubated in anti-rabbit anti-phospho Erk1/2, anti-phospho MEK1/2, anti-phospho JNK, anti-phospho p38, anti-phospho Akt, anti-Erk1/2, anti-
MEK1/2, anti-JNK, anti-p38, anti-β actin, anti-phospho STAT1 (1:1000; Cell Signaling), anti-IRF3 and anti-phospho STAT3 (1:1000, Abcam), anti-mouse anti-GAPDH, anti-phospho EGFR, and anti-HA tagged (1:1000, Santa Cruz biotechnology). Goat anti-rabbit and goat anti-mouse IgG-horseradish peroxidase conjugated secondary antibody (Promega) and enhanced chemiluminescence reagent (Sigma Aldrich) were used for visualization of immune complexes.

**Luciferase reporter assay.** Tandem ISRE elements were cloned upstream of the luciferase reporter gene (obtained from Dr. Jenny Ting). The ISRE-luc reporters were co-transfected with pCMVHA, pHPV16WT, pHPV16ΔE5, pCMV16E5 and pCMV6E5 in OKF6 cells. At 48 hours post-transfection, luciferase activity was measured using luciferase reporter reagent system (Promega) and was reported as relative light units.

**Immunofluorescence.** Immunofluorescence for p-STAT3, p-EGFR, ISG54 and HA-tagged protein were performed on OKF-6 cells grown and transfected with pCMVHA, pHPV16WT, pHPV16ΔE5, pCMV16E5 and pCMV6E5 for 48 hours on chamber slides. OKF6 cells were incubated in p-STAT3, EGFR, ISG54 and HA antibody (1:100, Cell Signaling) for 24 hours at 4°C. The secondary goat anti-mouse IgG AlexaFlour 488 and goat anti-rabbit IgG AlexaFlour 546 antibody were used to detect HA and p-STAT3, ISG54, p-EGFR proteins respectively. OKF6 cells were further incubated with 4’6’-diamidino-2-phenylindole (DAPI) for 5 minutes.

**Statistical analysis.** Kolmogorov-Smirnov was used for normality test. Mann-Whitney test was used to evaluate statistical differences between groups in abnormal distribution group. Student t-test was used in normal distribution group. The threshold for statistical significance was at P values <0.05.
Results

HPV E5 containing constructs were generated and expressed.

A full length HPV16 genome clone was generated from an oral cancer biopsy. The HPV16E5 amino acid sequence was identical to previously published results and demonstrates 10% amino acid homology with HPV6E5 (17) (Figure 2.1). pCMVHA vector (Figure 2.2A) that express the full length high-risk (HR)-HPV16 (HPV16WT), full length HPV16 with E5 deleted (HPV16ΔE5), HPV16 with a stop codon introduced in E5 (full length, pHV16E5stop and overexpression, pCMV16E5stop), and low-risk (LR)-HPV6E5 (pCMV6E5) overexpression constructs were generated. The schema for constructions of E5 mRNA expressing plasmids (Figure 2.2B), and for E5 deletion mutants in the context of the HPV16 genome is shown (Figure 2.2C).

**Figure 2.1 Amino acid sequence alignment.** Amino acid sequence alignment showed the differences between HR-HPV16E5 (bold) and LR-HPV6E5. Conserved amino acids between HR-and LR-HPVE5 are paired and shown by bold lines.

These were generated using standard PCR conditions and were sequence confirmed. After transfection of OKF6 oral epithelial cells with pHV16WT, E5 constructs, or empty vector, E5 mRNA expression was confirmed by real time RT-PCR normalized to GAPDH using HPV16E5
and HPV6E5 specific primers (Figure 2.2D). The expression of HPV16E5 in pHV16WT, pCMV16E5, pHPV16ΔE5, pCMV16E5stop, pHV16E5stop, and pCMV6E5 expressing cells compared to vector control were 9.9, 12.4, 2.3, 15.8, 29.6, and 81.9 fold, respectively. E5 protein expression was confirmed by Western blot and cytoplasmic expression was detected by immunofluorescence (Fig 2.2E, 2.3A and 2.3B) using anti-HA antibody. E5 HA-tagged protein was detected for both pHV16E5 and pHV6E5 although the c-terminal tagged E5 proteins were much more readily detected (Figure 2.2A and 2.3A-B lanes 4 and 8). Expression of an early HPV gene, E2 (Fig 2.2F), and a late HPV gene, E1^E4 (Figure 2.2G), in the context of the whole genome were confirmed by quantitative RT-PCR detection of these genes for HPV16WT, HPV16ΔE5 and HPV16E5stop constructs.

A.
Figure 2.2 HPV E5 constructs were generated. HPV E5 constructs were generated C-terminal HA-tagged, and expressed both mRNA and protein. A) pCMV vectors were inserted with 1) HPV16WT whole genome, 2) HPV16 genome with E5 deletion, 3) HPV16 with E5 transcribed but not translated, 4) HA-tagged HPV16E5 gene alone, 5) HA-tagged HPV16E5 transcribed but not translated gene alone, 6) HA-tagged HPV6E5 gene alone. B) E5stop codon construct with 2 stop codons inserted at codons 3 and 5. C) HPV16 with E5 ORF deletion construct with E5 specific deletion primer containing Pfimfi and Apal restriction sites. D) The expression of HPV16E5 in pHV16WT, pCMV16E5, HPV16∆E5, pCMV16E5stop, pHV16E5stop, and pCMV6E5 expressing cells compared to pCMVHA vector control was 9.9, 12.4, 2.3, 15.8, 29.6, and 81.9 fold, respectively. E) HA-tagged protein expression (green) in pCMV16E5 and pCMV6E5 expressing cells (63X magnification). F) HPV16E2 mRNA expression and G) E1^E4 mRNA expression detected by real time RT-PCR. E5, E2, E1^E4 mRNA expression were normalized to GAPDH and pCMV vector (2-ΔΔct.)
HPV16E5 induced p-MEK1/2, p-Erk1/2 and p-p38 MAPK signaling transduction compared to HPV6E5 in OKF-6 cells. To compare signal modulation in HPV16E5 and HPV6E5 expressing cells, p-MAPK and p-Akt expression were assessed by immunoblotting. HPV16E5 consistently induced higher expression MAPK family members, p-MEK1/2, p-Erk1/2, and p-p38 compared to HPV6E5 (Figure 2.3A lanes 3-5 vs lane 6-8). HPV16ΔE5 demonstrated a loss in p-MEK1/2 and p-p38 induction ability (Figure 2.3A lane 2). HPV16E5 suppressed p-Akt expression compared to HPV6E5, HPV16ΔE5, and vector control while p-JNK was unaffected by HPVE5 (Figure 2.3B). These results differ from a previous HPV16E5 study in cervical cell lines where HPV16E5 induced phosphorylation of Akt and Erk1/2, but not p38 and JNK (39). In oral keratinocytes, MAPK (p-p38, p-MEK1/2 and p-Erk1/2) was utilized by HR-HPV16E5 while LR-HPV6E5 activated Akt. This suggests cell type specific E5 function utilizing different signaling pathways.

To confirm differential pathway regulation by high- and low-risk HPVE5, pCMV16E5- and pCMV6E5-expressing OKF6 cells were treated with the p38 inhibitor SB202190 (SB). An anti-inflammatory drug Cox-2 inhibitor (Cox2i), Celecoxib, has been proved to inhibit p-p38 in recurrent respiratory papilloma (40) and was used as a second p-p8 inhibitor in this study (Figures 2.3C and 2.3D). SB targets active form of p-p38 while Cox2i targets Rac1, a signal transduction protein that acts in the linear EGFR pathway up-stream of p38 MAPK (40). A decrease in p-p38 was detected with SB treatment in both pCMV16E5 and pCMV6E5 expressing cells (Figure 2.3C). Cox2i decreased p-p38 protein expression in cells overexpressing pCMV16E5, but p-p38 levels in pCMV6E5 expressing cells were not affected (Figure 2.3D). Deletion of the E5 ORF in HR-HPV16 displayed differential results with SB and Cox2i, with inhibition of p38 phosphorylation by SB but not by Cox2i. E5 protein of LR-HPV6E5 did not utilize the p38 pathway. These data confirm HPV16E5 regulation of p38 MAPK modulation in oral keratinocytes.
Figure 2.3 HPVE5 differentially activates the p38 pathway in OKF6 cells. A) pCMV16E5 expressing cells showed higher levels of p-MEK1/2, p-Erk1/2, and p-p38 protein expression compared to pCMV6E5 expressing cells. HPV16ΔE5 lost the ability to induce p-MEK1/2, p-Erk1/2, and p-p38 protein expression. HA-tag protein expression represented E5 expression in pCMV16E5 and pCMV6E5 expressing cells. B) pCMV16E5 expressing cells demonstrated decreased expression of p-Akt, whereas comparable expression of p-Akt to empty vector was seen in HPV6E5 expressing cells. There was no difference between p-JNK with HR- or LR-HPVE5. C) p-p38 inhibitor SB202190, reduced p-p38 protein expression in pCMV16E5, HPV16ΔE5 and pCMV6E5 expressing cells. D) Cox2-inhibitor (Cox2i) reduced p-p38 protein expression.
expression only in pCMV16E5 expressing cells. Phospho-p38 levels were normalized to β-actin in A), B), and D), and were normalized to GAPDH in C). Densitometry shows the expression of p-p38 normalized to total p38 and GAPDH.

**High risk HPV16E5 enhanced the activation of type 1 interferon responses while low risk HPV6E5 was unable to modulate this response.** HPVE5 modulation of the type 1 interferon response in OKF6 oral epithelial cells was assessed. HPV16 E5 has previously shown to induce IFNβ in HaCaT cell and C33A cells (30), but the role of HPV16E5 and HPV6E5 on type I IFN responses has not been determined in oral cells before. To investigate the role of HPVE5 on the type I IFN response, a reporter construct containing tandem interferon stimulatory response elements (ISRE) was co-transfected with E5 constructs. A 2.5 fold difference in ISRE promoter activity was detected between pCMV16E5 and pCMV6E5 expressing cells (P=0.028) (Figure 2.4A). HPV16E5 induced ISRE activity at levels comparable to HPV16WT and HPV16ΔE5 levels were lower than HPV16WT. Interferon stimulated gene (ISG) transcripts for ISG54 and 2′5′OAS were examined by real time RT-PCR. pCMV16E5 demonstrated enhanced induction of both ISG54 (4.37 fold increase, P=0.026) and 2′5′OAS (3.60 fold increase, P =0.030) compared to pCMV6E5 (Figure 2.4B-C). Interestingly, the greatest up-regulation of ISG54 and 2′5′OAS expression occurred in cells expressing HR-HPVE5 protein, over expressed or in the context of the HPV16WT genome. These figures reflect three to five biological replicates, each performed in triplicate. In the presence of the Cox-2 inhibitor, HPV16WT and HPV16E5 mediated 2′5′OAS expression was down-regulated; HPV6E5 expression did not have this effect (Figure 2.4D). ISG54 expression was examined by immunofluorescence. Punctate nuclear staining for ISG54 was detected in HPV16WT, pCMV16E5 and positive control poly I:C and was detected at much lower levels in pCMV6E5 containing cells, comparable to vector alone (Figure 2.4E).
Figure 2.4 HR-HPVE5 expression in oral epithelial cells modulated interferon response in a p38 dependent manner. A) HPV16WT and pCMV16E5 induced significantly higher ISRE promoter activity compared to pCMV6E5 (2.5 fold), demonstrated by luciferase assay. B) pCMV16E5 induced significantly higher expression of ISG54 mRNA expression and C) 2’5’OAS mRNA expression compared to pCMV6E5. D) Cox-2 inhibitor reduced 2’5’OAS mRNA level in HPV16WT, pCMV16E5, HV16ΔE5, and pCMV6E5 expressing cells comparing to untreated cells and secondary antibody alone as a control. ISG54 and 2’5’OAS mRNA expression were
normalized to GAPDH and pCMV vector (2-∆∆ct). E) pCMV16E5 and HPV16WT demonstrated high ISG54 protein expression while the ISG54 expression in pCMV6E5 expressing cells was comparable to pCMV vector alone. ISG54 (red), HA-tagged (green), and DAPI (blue nuclei). 63X magnification.

STAT regulation by HPV has been described, often in the context of E6 and E7 (41, 42). Here, the potential for E5 mediated p-STAT3, p-STAT1, and IRF3 expression was assessed. Immunofluorescence determined that cells expressing HPV16WT, pCMV16E5 (green) or poly I:C demonstrated higher p-STAT3 (red) levels at the plasma membrane compared to pCMV6E5 (green), HPV16∆E5 expressing cells and vector control (Figure 2.5A). Likewise, immunoblot detected higher levels of p-STAT3 with HR-HPV16E5 expression compared to LR-HPV6E5 (Figure 2.5B and 2.5C). This is true at both 12 and 48 hours post transfection. Similar to 2’5’OAS mRNA expression (Figure 2.4D), pharmacologic inhibition of p-p38 with SB brought pCMV16E5 p-STAT3 protein levels down to levels detected in pCMV6E5 expressing cells (Figure 2.5B), suggesting that HPV16E5 mediated activation of ISGs was p38 dependent. For p-STAT1, immunoblot detected higher levels of p-STAT1 with HR-E5 expression compared to LR-E5 at 12 hours. However at 48 hours post transfection, HPV16E5 expression no longer increased p-STAT1 (Figure 2.5C lanes 3 and 7). pCMV16E5 and pCMV6E5 mediated similar levels of IRF3 at 12 hours and 48 hours. (Figure 2.5C lanes 3 and 7).
Figure 2.5 HPV16E5 regulates the expression of p-STAT1, p-STAT3 and IRF3. A) pCMV16E5 and HPV16WT demonstrated high p-STAT3 protein expression while the ISG54 expression in pCMV6E5 expressing cells were comparable to pCMV vector alone. STAT3 (red), HA-tagged (green), and DAPI (blue nuclei). 63X magnification. B) p-p38 inhibitor (SB202190) reduced p-STAT3 protein expression in HPV16WT, pCMV16E5, and HPV16ΔE5 treated OKF6 cells. Densitometry shows levels of p-STAT3 expression normalized to β-actin and GAPDH. C)
At 12 hours post transfection, pCMV16E5 induced higher levels of p-STAT1 and p-STAT3, but not IRF3, compared to pCMV6E5 expressing cells (left panel). At 48 hours post transfection, pCMV16E5 suppressed p-STAT1 while pCMV6E5 induced p-STAT1 expression in comparable levels to pCMV vector. The expression levels of p-STAT3 and IRF3 were higher in pCMV16E5 expressing cells compared to pCMV6E5 expressing cells (right panel).

**HPV16E5 induction of MAPK and ISG was EGF dependent in oral epithelial cells.** One possible pathway leading to MAPK activation and ISG expression is through phosphorylation of EGFR (19, 23, 43). EGFR phosphorylation was detected with expression of HR- and LR-pCMV5E, while p-EGFR in HPV16∆E5 expressing oral epithelial cells was comparable to control by both immunofluorecence (red) (Figure 2.6A) and immunoblot (Figure 2.6B lanes 2, 3 and 5). To determine whether pCMV16E5 mediated induction of MAPK and ISGs was EGF dependent, experiments were performed in the presence and absence of EGF. pCMV16E5 mediated phosphorylation of Erk1/2 occurred in the absence of EGF at 1.2 fold over vector control (Figure 2.6C lane 3). However the presence of EGF resulted in more robust Erk1/2 phosphorylation at 2.5 fold over control (Figure 2.6C lane 12). Interestingly, in the absence of EGF, constructs expressing just HPV16E5 mRNA (pCMV16E5stop) enhanced the expression of Erk1/2 (Figure 2.6C lanes 5 and 6). In the presence of EGF, Erk phosphorylation was mediated by HPV16WT and pCMV16E5 protein but not HPV16∆E5 or pCMV6E5 protein. However, the absence of EGF did result in loss of pCMV16E5 mediated activation of interferon stimulated genes, ISG54 (Figure 2.6D) and 2’5’OAS (data not shown). pCMV16E5 mediated ISG54 mRNA mean levels in the presence of EGF were 23 fold over vector control and were higher than the 19 fold increase detected with IRF7 expression. In the absence of EGF, pCMV16E5 mediated ISG54 levels were comparable to pCMV6E5 and to vector control. These results suggest that while HPV16E5 induced MAPK signaling was minimally EGF independent, ISG expression absolutely required EGF.
Given the significant activation of the MAPK pathway by HR-HPV16E5, even with little HA tagged protein detected (Figure 2.3A), the potential for HPVE5 mRNA to regulate MAPK and ISG expression was investigated. In the presence of EGF, HPV16E5 mRNA, independently and in the context of the HPV16 genome (pCMV16E5stop and pHPV16E5stop), was less effective than HPV16E5 protein (HPV16WT and pCMV16E5) at phosphorylating Erk1/2 protein (Figure 2.6C). Interestingly, HPVE5 mRNA demonstrated EGF independent phosphorylation of Erk1/2 that was not detected in the presence of EGF (Figure 2.6C). In the absence of EGF, HPV16E5stop mRNA was superior to both HPV16WT and pCMV16E5 in the induction of ISG54 (Figure 2.6C). In the presence of EGF, HPV16E5 mRNA induced comparable levels of ISG54 transcript compared to HPV16E5.

A.
Figure 2.6 HPVE5 expression in oral epithelial cells resulted in phosphorylation of EGFR and activation of Erk1/2. E5 mediated interferon responses were EGF dependent. A) pCMV16E5 and pCMV6E5 induced p-EGFR protein expression in comparable levels to HPV16WT. B) In EGF containing media, HPV16WT, pCMV16E5 and pCMV6E5 expressing cells showed higher levels of p-EGFR compared to HPV16ΔE5 expressing cells. C) In EGF-media, pCMV16E5, pCMV16E5stop and HPV16E5stop induced higher levels of p-Erk1/2 compared to pCMV6E5 and HPV16WT, which induced p-Erk1/2 in comparable levels to pCMV vector (left panel). In EGF+ media, HPV16WT and pCMV16E5 expressing cells induced higher levels of p-Erk1/2 compared to HPV16ΔE5, pCMV16E5stop, HPV16E5stop, and HPV6E5 expressing cells (right panel). Densitometry shows the expression of p-Erk1/2 normalized to total Erk1/2 and GAPDH. D) Real time RT-PCR showed the ISG54 induction ability compared between EGF- media (left panel) and EGF+ media (right panel). In EGF+, but not in EGF-, media ISG54 induction is significantly higher in pCMV16E5 expressing cells compared to
pCMV6E5 expressing cells (p<0.05) and in HPV16WT expressing cells compared to HPV16∆E5 (p<0.01). Student’s t-test was used for statistical analysis. *p<0.05, **p<0.01.

**HPV16E5 modulated HPV16 E6 and E7 oncogene expression in oral epithelial cells.**

Alternative splicing of HPV16 E6*/E6 early mRNA has recently been shown to be coupled to EGF signaling via Erk1/2 activation (44). Further, EGF depletion lead to both a higher E6*/E6 ratio and enhanced E7 expression (44). As HPV16E5 activated more robust p-Erk1/2 in the presence than in the absence of EGF (Figure 2.6), we hypothesized that HPV16E5 modulated E6 alternative splicing, potentially impacting the expression of both full length E6 and E7. Using HPV16E6 specific primers for real time RT-PCR, we detected the E6 spliced form (E6*) in the absence of E5 (HPV16∆E5 and HPV16E5stop) (Figure 2.7A lanes 3 and 5) but not in the presence of E5 in the context of the genome (HPV16WT) (Figure 2.7A lane 2). Quantitative RT-PCR detected a four-fold increase in the level of spliced E6* transcripts in the absence of HPV16E5 (HPV16∆E5) compared to HPV16WT expressing HPVE5. An intermediate phenotype was detected with the expression of E5 mRNA in the context of the HPV16 genome (Figure 2.7B). Consistent with an increase in splicing, an increase in the nuclear expression of HPV16E7 was observed by immunofluorescence in cells lacking E5 expression. With HPVE5 expression, lower E7 levels were detected by immunofluorescence (Figure 2.7C) under conditions with higher levels of full length E6 expression (Figure 2.7B).
Figure 2.7 HPV16E5 controls the expression of E6*/E6 and E7. A) cDNA extracted from HPV16ΔE5 and HPV16E5stop expressing cells showed a spliced E6 band at 300 kb. HPV16WT did not have spliced E6. B) Real time RT-PCR showed approximately four-fold higher E6 splicing in HPV16ΔE5 compared to HPV16WT. C) E7 protein expression was higher in HPV16ΔE5 compared to HPV16WT. IFA staining for HPV E7 (green) and DAPI (blue nuclei). 63X magnification. NS = non-significant.
Discussion

Proliferation and oncogenesis are key to the development of HPV associated oral lesion and malignancies. The role of HPVE5 in cellular proliferation and oncogenesis is largely attributed to manipulation of growth factor receptor pathways and their associated tyrosine kinases (19, 22, 26, 27). In this study, a direct comparison of HR-HPV16E5 and LR-HPV6E5 in oral epithelial cells determined that HR-HPV16E5 activated EGF regulated MAPK pathways that induced the interferon response and modulated E6/E7 expression. Expression of LR-HPV6E5, however, resulted in minimal type 1 interferon regulation.

HR- and LR- HPVE5 modulate MAPK signaling by prolonging EGFR cell surface expression (17, 19, 22, 45). The 5’ carboxyl terminal amino acids of E5’s third hydrophobic domain are important to E5 mediated EGFR activation and downstream induction of matrix metalloproteases (MMPs) (46). The carboxyl terminal amino acids are not conserved between HR- and LR-HPVE5, perhaps explaining the differences that were detected in EGFR mediated signaling between HR- and LR-HPVE5 in this study of oral keratinocytes. As previously shown in cervical cells, both HR-HPV16E5 and LR-HPV6E5 increased the phosphorylation of EGFR (Figure 2.6 A and B) (18, 19, 27). HPV16E5 has previously been shown to up-regulate VEGF expression through activation of both p-Akt and p-Erk1/2 in HaCaT cells (26, 47). In this study of oral epithelial cells, HPV16E5 and HPV6E5 activated different pathways. p-Akt was detected in HPV6E5 expressing cells but was suppressed by HPV16E5 (Figure 2.3). It was determined that while HPV6E5 expressing cells did not stimulate p-Erk1/2 and p-p38 expression, HPV16E5 expressing cells (HPV16WT and HPV16E5) did. MAPK activation was largely EGF dependent (Figure 2.6) and was confirmed by pharmacologic perturbation targeting both Cox-2 and p38 (Figure 2.3). Wild type HPV16 with E5 deleted demonstrated a MAPK modulation profile similar to LR-HPV6E5. In the absence of EGF, HPV16E5 lost p-Erk1/2 induction ability, behaving similarly to HPV6E5. p-JNK expression was not affected by either HPV16E5 or HPV6E5.
The type I IFN response is the first line of defense against viral infection in human keratinocytes (33). HPV-positive OSCCs exhibit high levels of type I IFN stimulated-gene expression (ISGs) (48). Interestingly, HR-HPV E6 and E7 directly interact with components of the interferon signaling pathway and down-regulate IFN-α, -β, and ISGs expression (33, 49). In our study, E6 and E7 expressing conditions, even in the absence of E5 (HPV16ΔE5), also demonstrated lower levels of ISG54 and STAT3 at the protein level (Figure 2.4 and 2.5). In this study, HR-HPV16E5 consistently activated members of the type 1 antiviral response while LR-HPV6E5 did not. Likewise, a recent study showed that HPV16E5 activated beta interferon and interferon regulatory factor 1 (30). Enhanced ISRE promoter activity, down-stream interferon stimulated gene transcripts (54 and 2′5′OAS) and ISG54, STAT1, and STAT3 proteins were detected in HPV16WT and pCMV16E5 expressing cells but not pCMV6E5 and HPV16ΔE5 (Figure 2.3-5). pCMV16E5 induced responses comparable to or higher than those induced by the entire HPV16 genome (HPV16WT). In previous studies, p38 MAPK was shown to act as a STAT1 serine kinase and cooperate with STAT1 to regulate IFN-β production and activate other type I IFN responses (35). Interestingly, our study showed that STAT3 expression was high in HPV16E5 expressing oral keratinocytes. STAT3 has been associated with tumor formation and tumor progression. Tissue biopsy from cervical cancer lesions showed a correlation between HPV16/18 expression and STAT3 expression (50). Along with this cervical study, a head and neck cancer study also demonstrated the overexpression of STAT1 and STAT3. Riebe et al, demonstrated the link between p-p38 and STAT3 by using p-p38 siRNA to down-regulate p-p38 and found decreased expression of STAT3 without any effect on STAT1 (51). In this study, we link E5 mediated signal transduction and the type 1 interferon response. ISG54 and STAT3 expression were p-p38 dependent in HPV16E5 expressing cells, as confirmed by Cox2 and p38 inhibitor experiments. E5 mediated ISG induction was EGF dependent as well (Figure 2.6). HR-HPVE5 was more effective at both signaling and induction of innate immune responses than LR-HPVE5 (Figure 2.8A-B). In the context of infection, modulation of IFN responses by HR-
HPVE5 may be temporally different than regulation by E6/E7. The HPV16ΔE5 demonstrated that E6/E7 expression in the absence of E5 consistently differentially regulated the expression of innate immune genes. Since STAT1 acts as a tumor suppressor while STAT3 is more attributed to tumor promotion, HR-HPV16E5 may utilize p-p38 induced STAT3 expression to promote tumor formation and progression in the head and neck area while the LR-HPV6E5 does not possess this function (Figure 2.8A-B).
Figure 2.8 Model of differential signaling mediated by HR and LR HPVE5, leading to benign or malignant disease. Cell proliferation and activation of intracellular immune responses are critical to the transformation of oral keratinocytes and subsequent cancer development. A) HR-HPV16E5 increased MAPK proliferation signals (p38, MEK1/2, and Erk1/2) and enhanced type I interferon responses (IFN promoter and ISGs), B) whereas LR-HPV6E5 induced small p-p38 response but high p-Akt. Both HR-HPV16E5 and LR-HPV6E5 induced MAPK by EGFR modulation. HR-HPV16E5 but not LR-HPV6E5 induced type I IFN responses via p-p38 and EGF induction. In addition, HPV16E5 plays an important role in E6* control. Since E6* is important for E7 transcription during the cellular differentiation process, E5 may indirectly control E7 oncogene expression. HPV16E5 mediated cell proliferation, intracellular immune responses, and E6/E7 expression may be critical for transformation of oral keratinocytes and subsequent cancer development.

HR-HPVE5 mediated activation of the interferon response through p38 may be critical to HPV oncogenesis. In this study, we show that both HR-HPV16E5 protein, and to a lesser extent HPV16E5 mRNA alone, could induce IRF3, ISGs, STAT1 and STAT3 in oral keratinocytes. LR-HPVE5 was unable to stimulate this response. In previous studies, exogenous IFN-β treatment resulted in rapid loss of HPV16 viral episomes with a simultaneous increase in the transcription of integrants (52, 53). Thus, it was postulated that the elimination of episomal virus by the interferon response was involved in the selection of HPV integrants (52). Given these important findings, we now suggest that E5 mediated p-38 induction of ISG expression could be instrumental to integration, an important early event in oncogenesis.

Also important to oncogenesis, we show that E5 modulates expression of other viral oncoproteins. It was previously suggested that IFN expression during HPV infection may abrogate episomal E2 protein expression resulting in high levels of E6 and E7 expression, thus accelerating cancer progression (53). In this study, E5 mediated interferon expression may
provide an indirect mechanism for E6/E7 oncogene modulation. E5 increased full length E6, diminished spliced E6 and decreased E7 expression (Figure 2.7). E6 splicing and the generation of E6* early mRNAs have previously been coupled to EGF signaling via Erk1/2 activation in cervical cells (44). Here, we determined a role for E5 in transcription of E6*/E6 by showing that E6* expression was detected in HPV16ΔE5 and in HPV16E5mRNA expressing cells but not in HPV16WT expressing cells containing E5. The lack of E5 resulted in a 4-fold increase in E6* and an increase in E7 expression. E5 mediated control of E6/E7 expression in partially differentiated oral epithelial cells suggests that E5 may act as a critical regulatory switch.

In sum, HR-HPV16E5 increased MAPK proliferative signals (p38, MEK1/2, and Erk1/2) and enhanced type I interferon responses (IFN promoter and ISGs) in an EGF dependent manner whereas HR deleted for E5 and LR-HPV6E5 were comparable to control. E5 mediated cell proliferation, activation of intracellular immune responses, and modulation of E6/E7 may be critical to the transformation of oral keratinocytes and subsequent cancer development. Future studies are needed to assess the role of E5 in integration and tumorigenesis.
REFERENCES


CHAPTER 3: HPV E5 SUPPRESSES DIFFERENTIATION AND ENHANCES PROLIFERATION IN ORAL KERATINOCYTES

Abstract

Human papillomaviruses (HPVs) cause significant morbidity and mortality in human. High-risk (HR) subtypes are highly associated with oropharyngeal carcinoma while low-risk (LR) subtypes are related to oral warts formation. HPV amplification is tied to epithelial differentiation with low-level of replication in basal layers and high copy numbers in supra-basal epithelium. Cell differentiation and activation of intracellular immune responses are critical to transformation of oral keratinocytes and subsequent carcinoma development. We hypothesized that HPV delayed terminal differentiation and modulated innate immune responses in oral epithelia. We investigated the role of HR-HPV16E5 and LR-HPV6E5 on oral keratinocytes differentiation and proliferation. pCMV vectors containing: whole genomes with E5 (HR-HPV16) or deleted E5 (HPV16ΔE5), HPV16E5-HAtagged (pCMV16E5), and LR-HPV6E5 (pCMV6E5) genome were generated. Constructs were transfected into primary foreskin keratinocytes and immortalized oral keratinocytes. Organotypic raft cultures were generated. Sodium butyrate and calcium chloride were used to induce differentiation. Differentiation, proliferation, E5HA, ISG54, IRF3, STAT1 and STAT3 were assessed by immunohistochemistry, IFA and real-time PCR. T-tests were used to determine statistical analysis. Lower levels of cross-linked involucrin were detected in differentiated cells containing HPV16WT and HPV16E5 but not HPV16ΔE5 and HPV6E5. In partially differentiated oral cells, HPV16E5 and HPV16WT induced Erk1/2 -phosphorylation, STAT3 and ISG54 protein expression while LR-HPV6E5 did not. At terminal differentiation,
HPV16E5 lost ISG54 and phospho-Erk1/2 induction ability and was similar to HPV6E5 and HPV16ΔE5 (P>0.05). Enhanced proliferation was detected in raft cultures expressing HPV16E5, HPV6E5 and HPV16WT cells compared to control. In conclusion, prolonged cell proliferation allows for higher rates of viral vegetation. HR-HPV16E5 delayed differentiation, induced phospho-Erk1/2 and induced innate immune genes whereas LR-HPV6E5 and HPV16ΔE5 were comparable to vector control. This study determined an important role for HR-16HPVE5 in early step of oral cancer progression.

Introduction

Human Papillomaviruses (HPVs) are small 8,000 kb double stranded DNA viruses infected in human cutaneous and mucosal epithelium (1, 2). Persistent human HPV infection can manifest as benign hyper-proliferative lesions or progress to malignancy (3). Of the over 150 HPV types, the major high risk (HR-) HPVs types 16, 18 are associated with cervical squamous cells carcinomas (SCCs) and are detected in oropharyngeal squamous cell carcinomas (OSCCs). Similar to HPV pathogenesis in cervical cancer, HPVs transform normal oral epithelial and are major drivers of head and neck squamous cell carcinomas (HNSCCs) in non-smokers (4-6). HNSCC is the sixth most prevalent human cancer worldwide with an estimated 405,000 new cases each year (7, 8). From 1988 to 2004 in the United States, tobacco and alcohol related cancers declined by 50%, but HPV-positive OCSSs increased by 225% (9). HPV16 is the major type detected in HPV-positive OSCCs (86.7%) and is more dominant in HPV-positive OSCCs (85%-95%) than in cervical cancer (50%-60%) (6, 10, 11). Interestingly, LR-HPV type 6 has been detected (2.5%) in OSCCs (6). Low risk HPV types, including types 6, 11, are most often found in genital warts, oral warts, and respiratory papillomas (1, 12). LR-HPVs associated oral warts have increased among HIV patients on highly active antiretroviral therapy (HAART) (13).

The HPV life cycle is tightly dependent on epithelial cells differentiation status. HPVs initially infect the basal cell layer where episomal viral genomes are maintained at low copy
number (50-100 copies per cell). In these undifferentiated cells, viruses require the expression of E1 and E2 protein together with S-phase cellular DNA machinery for viral replication (14). HR-HPVs genomes encode five non-oncogenic proteins and three oncoproteins; E5, E6, and E7. HR-HPV oncoproteins alter cell apoptosis, induce immortalization and transform normal cells (15-17). Both HR- and LR-HPVs E5, E6, and E7 induce cell proliferation. As cells ascend toward the epithelial surface, HPVs undergo productive replication where copy numbers increase to over 1,000 copies per cell. In supra basal cells, the late promoter controls expression of late HPV proteins E1^E4, E4, L1 and L2 and high levels of E5 expression are detected. Despite the conserved genome organization and viral life cycle between HR- and LR-HPVs, only HR-HPV genomes produce oncogenic E5, E6, and E7. Integration and the binding affinity to cell cycle regulatory proteins are properties unique to HR-HPVs (12). HR E6 and E7 interrupt cell cycle, damage host DNA, induce cell transformation and suppress IRF-3 and IRF-7 (14, 18-22). While HPV16E6 and E7 alter cell differentiation in human foreskin keratinocytes (23), HPV16E5 mutants did not alter keratinocyte differentiation and did not affect the formation of virus like particles (24).

HPV E5 is a hydrophobic protein localized to the endoplasmic reticulum and Golgi apparatus (25). HPVE5 is highly expressed in supra-basal cell layers, suggesting that HPVE5 expression is tightly associated with cell differentiation. Although HR- and LR-HPVE5 share few identical amino acids, both have three hydrophobic functional domains that perform similar functions including cell proliferation, activation of growth factor signal transduction and binding of MHC Class1 (26). The first domain (27) of both HR- and LR-HPVE5 interacts with and inhibits MHC class I expression on the cell surface (26, 28). The first and second hydrophobic domains bind to the 16-kDa subunit of vacuolar ATPase, block endosomal acidification, and reduce EGFR degradation in human keratinocytes (27). The third hydrophobic domain of HPV16E5 binds to the B-cell associated protein 31 (Bap 31), an exporter protein that carries MHC class I molecules out of the ER (29). HPV16E5 also binds to the perinuclear small transmembrane lipoprotein A4 (30). A4 has been found to interact with Bap31, hence, HPVE5 may form a complex with Bab31 and
A4 to control cell proliferation and late viral gene expression (29). Prolonged proliferation of human foreskin keratinocytes was detected in association with HPV31E5 binding to Bap 31 (29). HR- and LR- HPVE5 proteins share similar functions related to cell proliferation. HPV16E5 elevates cell surface levels of EGFR, increasing recycling and activation of EGFR (26-28, 31, 32). In the presence of EGF, both HR- and LR-HPVE5 up-regulate the phosphorylation of MAPK signaling molecules p-Erk1/2 (26, 33, 34). HPV16E5 transfected cells demonstrated enhanced phosphorylated-Erk1/2 and PI3K/Akt (35). Both HPV16E5 and HPV6E5 induce rodent fibroblast transformation in soft agar (16, 36) and induce koilocyte formation, a morphological marker of HPV infection (37). However, HPV16E5 exhibits higher mitogenic activities compared to HPV6E5 (16, 36) and only HPV16E5, not LR-HPV6E5, up-regulates VEGF expression through EGFR (35) and inhibits TRAIL and FasL mediated cell apoptosis (38).

HPV E5 is important to viral DNA synthesis and controls cell differentiation during productive infection (24). The role of E5 particularly in oral epithelial cells in the context of oropharyngeal cancer has not been investigated and remains unclear. Recent studies determined a role for high-risk E5 interferon stimulated gene (ISGs) expression (33). The purpose of the present study is to determine the effect of HR-HPVE5 and LR-HPVE5 on oral epithelial cell differentiation, proliferation, and modulation of the type-1 interferon responses.

Materials and Methods

Cells culture. Primary human foreskin keratinocytes (HFKs) were grown in KSF media supplemented with 0.2 ng/ml EGF, 25 mg/ml BPE, 0.01% penicillin-streptomycin and 10% ITA. The telomerase immortalized oral keratinocyte cell lines (OKF-6) were grown in the KSF media supplemented with 0.2 ng/ml EGF, 25 mg/ml BPE, penicillin-streptomycin and 0.4 mM CaCl₂. Cells were grown in media for 24 hours before transfection with HPV16WT, empty vector, pIRF-7 and E5 constructs. To induce differentiation, cells were further treated with 0.6 M Sodium Butyrate (NaB) or 1.5 μM CaCl₂ for 48 hours before harvesting with Trizol (Invitrogen).
**HPVE5 DNA constructs and transfection.** HPV16WT and HPV6WT were used as a template to establish constructs expressing the full length HPV16 genome (HPV16WT), the full length HPV16 genome with deletion of the E5 gene open reading frame (pHPV16ΔE5), HA tagged overexpression constructs containing HPV16E5 (pCMV16E5) and HPV6E5 (pCMV6E5) (Figure 3.1A-C). The full length HPV16 genome was PCR amplified from oral cancer biopsy DNA using a Roche Expand Long Template PCR System (Roche) and the primers HPV16BamHIF; 5′-CCCGGATCC CCATGTACCAATGTTGCA-3′ and HPV16BamHIR; 5′-CCCGGATCC TTTGCCCCAGTGTTCC-3′. The 7.9 kb PCR fragment was TA-cloned into pCMV cloning vectors. To overcome the poor immunogenicity of the E5 protein, E5 expression plasmids were tagged with hemagglutinin (HA) epitope at the C-terminus and designated as pCMV-HA. We generated different E5 constructs by using Taq polymerase (Invitrogen) and standard PCR condition with following E5 primers: 1) HPV16 wild type genome, 2) HPV16E5 overexpression constructs with the N-terminal HA- tagged (pHPV16E5): 5′-GCGAAGCTTACC ATGTACCCATACGATGTCCAGATTACGCTATGACAAATCTTGATcTCGCGTCTATGAAATCTGAACTTACTAGAATCAGCTGGAAC-3′ and 5′-GCG CTGAGTTATGTAAATTTAAAGCGTGCACTGACGTTACAAT-3′, 3) HPV16 genome with deletion of the E5 ORF using specific primer for E5 deletion (pHPV16ΔE5): 5′-TTACAGTGTCTACTGGATTTATGTCTATGAAATAAACTGTTACT-3′ and 5′-CGCATTTAGTAAAGCTGCGTCTGAAAGCTGGAAC-3′, 4) HPV6 E5 with HA-tagged (pHPV6E5): 5′-GCGAAGCTTACC ATGTACCCATACGATGTCCAGATTAGCTGGGAAATCGTACTTTTGTA TGA-3′ and 5′-GTGATCAAAGCTGCACTGTTACCCAGTTACCAATCTGAAAGCTGGAAC-3′, and 5) pCMV containing IRF-7 ORF (pIRF7). The deletion HPV16E5 plasmids were established by using specific deletion primers for E5 with standard PCR protocol deletion at PfiMI and Apal restriction site. The PCR product maintained first 5 amino acid of HPV16E5 ORF where overlapped to stop codon of HPV16E2 for normal E2 protein transcription and translation. Each set of primers carried HindIII and Xhol sites to enable cloning into restriction endonuclease sites in pcDNA3 by using T4 DNA ligase.
Plasmids were cloned and selected in DH5α Escherichia coli (Invitrogen) following company instruction.

For transfection, OKF6 cells were grown to 70% confluence and transient transfected with pHV16, pCMV, pIRF, and different E5 constructs using Fugene-6 (Roche). pRFP was co-transfected for transfection efficiency determination. Cells were allowed to grow for 48 hours before harvesting for protein and RNA. E5 mRNAs expression were measured by Real-time RT-PCR using the following E5 primers; HPV16E5 5'- CATCCACAACATTACTTGCGTGCT-3' and 5'-CGAGAGGCTGCTGTATCCACAAT-3', HPV6E5 5'- GGAAGTGTTACCTGTACAAATAGC TG-3' and 5'-GGGTTGTTAATAGCAGCCACA-3' normalized to GAPDH.

**Western Blotting.** After 48 hours of NaB treatment, HFKs and OKF-6 cells were lysed with Ripa buffer for protein lysate. Lysates were centrifuge at 10,000 rpm for 10 minutes. Supernatants were collect and were analyzed for protein concentration by using Biorad Protein Assay Kit. Protein lysates were electrophoresed through 4-12% sodium dodecyl sulfate (SDS)-polyacrylamide gel (Life Technologies, Inc.) and transferred to nitrocellulose membrane (Whatman). Membranes were blocked with 5% non-fat dry milk for 1 hour and incubate with 1:1000 anti- rabbit IgG antibody for phospho-Erk1/2, phospho p38, Erk1/2, p38, HA tagged, involucrin and beta actin (Cell signaling). 1:10000 Anti-rabbit IgG-horseradish peroxidase (Promega) was used as a secondary antibody and enhanced chemiluminescence reagent (Invitrogen) was used for immune complexes identification followed manufacturer’s recommend.

**Real-time RT-PCR.** At 48 hours post-NaB or post-CaCl2 treatment, cells were washed twice with PBS. Total RNA extracts were isolated using Trizol (Life Technologies, Inc.). 1 μg of RNA was reverse transcribed to cDNA by SuperScript III (Invitrogen) and oligo-(dNTP) primed. Real-time reverse transcription-PCR (real time RT-PCR) was performed in a LightCycler instrument with cDNA master SYBR Green I dye (Roche Molecular Biochemicals). Real time RT-PCR for type I interferon stimulated gene expression was performed with ISG54 specific primers: ISG54F 5'
TTCACCTCTGGACTGGCAATAGCA-3’ and 5’-AAGCTTCAGAGCCAGGAGGACTT -3’; and 2’-5’ OAS: 5’-GTTGGTGTTGGCATCTTCTGGCAA-3’ and 5’- TTGGGTTTCCAGTCCC ATCCTTGAA-3’. Real time RT-PCR for K1 was performed with K1 primers: hKer1F 5’-GCAAAACAAAATGGGAGCTG-3’ and hKer1R 5’- GCTCTAAATTATGGGTCTAGTGGA -3’. GAPDH was used as an internal control. PCR condition was as followed: initial denaturation at 95°C for 10 minutes followed by 50 cycles of alternating at 95°C for 10 seconds, annealing step at 50°C for 10 seconds and the extension step at 72°C for 30 seconds. The cDNA amplification was performed in triplicate and the ISGs gene expressions were compared to empty vector pCMVHA before normalized to GAPDH (2-ΔΔCt).

**Organotypic raft culture:** 2x10^5 cell/ml human foreskin fibroblasts (J2) were embedded in 1 ml of rat tail collagen type I (4.5 mg/ml; Fisher Scientific) and coated on trans-well insert (24 mm in diameter with 0.4 μm pore size; Costar). The trans-well inserts with J2-collagen were incubated in DMEM media contained 10% Bovine calf serum and 0.01% penicillin-streptomycin at 37°C in 5% CO₂ incubator for 48 hours. Before growing OKF6 on J2-collagen dermal equivalent, trans-wells were washed twice with PBS then changed media to KSF media supplemented with 0.2 ng/ml EGF, 25 mg/ml BPE, 0.01% penicillin-streptomycin and 10% ITA. Two days after epithelial cell plating, media was removed and cells were feed from the bottom of the trans-wells to allow the air-liquid interface. Media was changed every other day. Fourteen days after air-liquid interface, rafts were lifted up and fixed with 4% paraformaldehyde for 15 minutes, washed with 70% ethanol, and subsequent embedded in paraffin. Paraffin blocks were cut into 4 μm thick sections that were stained for H&E and antibodies for Involucrin, (Santa Cruz), K10, HA, p-STAT1 (Cell signaling), p-STAT3, p-IRF3, ISG54, and filaggrin (Abcam)

**Immunohistochemistry:** The paraffin-embedded raft sections were de-paraffinized in xylene for 5 minutes 2 times and rehydrated in gradient alcohol series. Antigen retrieval was performed in 10mM sodium citrate buffer for 20 minutes at 70°C followed by quenching with 3% H₂O₂ in methanol for 20 minutes at room temperature. Slides were blocked with 2.5% normal goat serum
in 0.1%BSA for 20 minutes before primary antibodies incubation; anti-mouse Involucrin (1:50), anti-mouse K10 (1:50), anti-mouse HA (1:50), anti-rabbit p-STAT1 (1:500), anti-rabbit p-STAT3 (1:100), anti-rabbit p-IRF3 (1:400), anti-rabbit ISG54 (1:250), and anti-rabbit filaggrin (1:50) for 24 hours at 4°C. All antibodies were detected using DAKO LSAB2 System Peroxidase kit (DAKO) followed manufacturer’s protocol. Briefly, slides were incubated in biotinylated reagent for 10 minutes then linked with streptavidin-HRP for 10 minutes. Substrate-chromogen solution was applied to slides for 5-10 minutes. All slides were counterstained with hematoxylin for 2 minutes, dehydrated in gradient alcohol then covered by DPX mounting media (Sigma-Aldrich).

**Immunofluorescence:** Immunofluorescence for HA-tagged protein was performed on OKF-6 cells grown and transfected with pCMV16E5 and pCMV6E5 for 24 hours in chamber slides. OKF-6 cells were fixed with 1.5% paraformaldehyde for 15 minutes at room temperature before permeabilized with 0.3% Triton-X in PBS. Chamber slides were then blocked with 1% goat serum and subsequently incubated in HA primary antibodies (1:100; Cell Signaling) for 24 hours at 4°C. The secondary antibody AlexaFlour 488 was used to detect E5HA protein. OKF6 cells were incubated in secondary antibody for 1 hour followed by 4’6’-diamidino-2-phenylindole (DAPI) for 5 minutes at room temperature then covered with Vectashield mounting media (Vector laboratories).

**Statistical analysis.** Student’s t test was used to evaluate statistical differences between means. Statistically significant was considered at P values ≤ 0.05.
Results

E5 expression in primary and oral epithelial cells results in hyperproliferation and detection of koilocytes. To determine and compare the influence of HPV16E5 and HPV6E5 on cell differentiation, E5 constructs were generated as described in materials and methods (Figure 3.1A). Briefly, HPV16WT was used as a template to create constructs expressing the full HPV genome with E5 (HPV16WT) or without E5 (HPV16ΔE5). HA tagged overexpression constructs were made for HPV16E5 and HPV6E5. Primary foreskin keratinocytes (HFKs) and oral epithelial cells (OKF-6) were transiently transfected with different E5 constructs and E5 mRNA expression was confirmed by real time RT-PCR (Figure 3.1B). Cytoplasmic expression of HPV16 and HPV6 HA-tagged E5 proteins expression was detected in cell monolayers by immunofluorescence (green)(Figure 3.2B). Raft cultures reminiscent of epidermis have been successfully used to investigate epithelial cell HPV infection. Cytoplasmic expression of HPV16 and HPV6 HA-tagged E5 proteins expression was detected throughout the strata of the organotypic raft by immunohistochemistry (Figure 3.1C).

Cellular consequences of proliferation were assessed in the context of E5 expression. Primary cells were rafted and assessed early in the process of differentiation where few cell layers had been established. Morphological changes were determined by hemotoxylin and eosin (H&E) staining. HPV16 WT expressing cells were hyperproliferative, invasive, and demonstrated koilocytes. As previously described, expression of both high and low risk E5 demonstrated koilocytes (37). Differentiated multilayer organotypic rafts of OKF6 cells expressing HPV16WT, deleted for HPV16E5 and overexpressing high and low risk E5 demonstrated a hyperproliferative phenotype with a thicker multilayer raft compared to control (Figure 3.2A). Organotypic rafts containing HPV16WT and HPV16E5 demonstrated spongiosis and epithelial cell maintenance of nuclei throughout the supra-basal cell layer, while rafts containing un-transfected cells showed less nuclei in supra basal cell layers consistent with terminal differentiation.
**Figure 3.1** HPVE5 expression A) pCMV vectors for transfection included 1) pCMV16E5HA, 2) pCMV6E5HA, 3) HPV16WT, 4) HPV16ΔE5. B) The expression of HPV16E5 transcripts with pHV16WT, pCMV16E5HA, HPV16ΔE5, and pCMV6E5HA in OKF6 cells compared to pCMVHA vector control. C) HPVE5 expression. HA-tagged protein expression in pCMV16E5HA and pCMV6E5HA expressing cells was detected by IFA – green staining (63X magnification) and by immunohistochemistry – brown staining (40x magnification).

Mitogenic pathways associated with cell proliferation were assessed. In undifferentiated primary cells immunoblot detected higher p-Erk1/2 and p-p38 expression in HR-HPV16E5 compared to LR-HPV6E5 expressing cells. HPV16E5stop and HPV16ΔE5 induced low levels of p-Erk1/2 and p-p38 levels that were comparable to empty vector. Differentiation was induced by adding 1.5 μM CaCl₂ to the cell culture media. Overall, little E5 mediated p38 phosphorylation was detected in undifferentiated cells but this activity increased substantially with differentiation. Interestingly, HPV6E5 demonstrated higher levels of p-Erk1/2 expression in undifferentiated primary cells compared to CaCl₂ treated cells (Figure 3.2B). Generally, higher p-Erk1/2 and p-p38 levels were detected in HPV16E5 expressing cells compared to HPV6E5 and HPV16ΔE5. Neither HPV16E5 nor HPV6E5 induced p-Akt under undifferentiated conditions. With CaCl₂ treatment, HPV16E5 and HPV6E5 induced comparable levels of p-Akt (data not shown). Taken together, these results indicated that HPV16 E5 activation of proliferation associated MAPK pathways required some level of differentiation (Figure 3.2B).
**Figure 3.2 HR-HPVE5 enhanced hyperproliferation, activation of proliferative signal transduction pathways, and acquisition a koilocyte phenotype.** Upon differentiation of primary cells LR-HPV E5 was no longer able to phosphorylate Erk1/2 while the HR-E5 can phosphorylate both p-p38 and p-Erk1/2 in undifferentiated cells and upon differentiation. A) HFK cells were grown on top of J2 cells in the organotypic culture. Cells were left to stratified for 14 days before fixed, paraffinized and stained by H&E staining. HPV16, HPV16E5 and HPV6E5 showed cell proliferation and cell invasion into collagen layer. All HPV16 infected cells showed the koilocytes displayed by the large perinuclear vacuole, acentric hyperchromatic nucleus. The
deletion of E5 results in minimal proliferative activity and abrogated koilosis. B) HPVE5 induced MAPK signaling was cell differentiation dependences. Western blotting showed the higher level of E5 induced p-Erk1/2 and p-p38 in partially differentiated stage comparing to non-differentiated stage. HPV6E5 showed the high level of p-Erk 1/2 at non-differentiated stage and less amount of p-Erk1/2 in differentiated stage, but higher level of p-p38 in partially differentiated stage.

**HR and LR E5 enhanced spinous differentiation.** Markers of spinous differentiation, Keratin1 (K1), Keratin 10 (K10), and involucrin, were assessed in E5 containing primary and oral epithelial cells. K1 mRNA was not detected in the absence of CaCl$_2$ (Figure 3.3A). However, in the presence of CaCl$_2$, HPV16 WT, HPV16E5 and HPV6E5 demonstrated 13, 6 and 11 fold increases in K1 mRNA expression, respectively. HPV16ΔE5 expressed the lowest levels of K1 at 3 fold increase. K10 and involucrin are also markers of cell differentiation, normally detected in supra-basal cell epithelium. In organotypic rafts, K10 was expressed throughout the strata of HPV16 and HPV16E5 over expressing cells and was detected at much lower levels in HPV16ΔE5 expressing cells. HPV6E5 K10 levels were comparable to HPV16ΔE5 (Figure 3.3B). Involucrin was detected at highest levels in HPV16WT expressing raft cultures. Both HR-HPVE5 and LR-HPVE5 demonstrated a gradient of involucrin expression with highest expression levels detected in the upper third of the raft. HPV16ΔE5 involucrin expression was comparable to control (Figure 3.3C). In sum, E5 enhanced spinous differentiation as determined by increased expression of K1, involucrin, and K10.
Figure 3.3 HR-HPVE5 enhanced spinous differentiation and delayed terminal differentiation. HR-HPV16E5 enhanced spinous differentiation determined by A) K1 and B) K10 expression. C) OKF6 was partially differentiated marked by monomeric involucrin.
expression (40X magnification). A) un-transfected control, B) HPV16WT, C) HPV16ΔE5, D) pCMV16E5HA, and E) pCMV6E5HA transfected cells.

**HPV16E5 delayed oral keratinocyte terminal differentiation in oral epithelial cells but HPV6E5 and HPV16ΔE5 did not.** The effect of HPV16E5 and HPV6E5 on terminal differentiation was assessed in oral keratinocytes. α-involucrin is expressed in two forms: a monomeric form expressed in partially differentiated cells and a cross-linked form expressed in terminally differentiated cells. Detection of the monomeric form in untreated OKF6 cells suggested that these cells were partially differentiated. OKF6 cells were induced to terminal differentiation using NaB as previously shown (un-published data). Densitometry determined at least two fold lower levels of cross linked α-involucrin detected by immunoblot in HPV16WT and HPV16E5 expressing cells compared to pCMVHA empty vector, HPV6E5, and HPV16ΔE5 expressing cells (Figure 3.4A). Fillagrin, a marker of terminal differentiation, was also assessed for expression in oral keratinocyte rafts. Lower levels of fillagrin staining were detected by immunohistochemistry in HPV16WT and HPV16E5 expressing cells compared to un-transfected cells, HPV6E5, and HPV16ΔE5 transfected (Figure 3.4B). Taken together these data suggest that HR E5 suppresses terminal differentiation in OKF-6 cells.
B. Filaggrin

Figure 3.4 HPV16WT and HPV16E5 altered oral keratinocyte terminal differentiation. A) OKF6 cell lines were treated with NaB to induce terminal differentiation. Cross-linked α-involucrin protein was a marker of cell differentiation. Protein lysates from pCMVHA, HPV16, HPV16E5, HPV16ΔE5, and HPV6E5 transfected OKF6 cells were separated on 4-12 % polyacrylamide gel. Proteins were transferred to nitrocellulose membrane and probed with α-involucrin antibody (1:1000). HPV16 and HPV16E5 showed less cross-linked α-involucrin protein expression comparing to HPV16ΔE5, and HPV6E5. The densitometry showed fold change of protein expressions normalized to pCMVHA. B) OKF6 was terminal differentiated
marked by filagrin expression (40X magnification). A) un-transfected control, B) HPV16WT, C) HPV16ΔE5, D) pCMV16E5HA, and E) pCMV6E5HA transfected cells.

**Upon differentiation E5 loses the ability to modulate the interferon response.** We recently determined that HPV16E5 more efficiently activated interferon stimulated gene expression than LR-HPV6E5 in OKF6 cell lines (submitted). Here, we determine whether HPV16E5 induced interferon responses are cell differentiation dependent. OKF6 cells were rafted and immunohistochemical studies were performed for ISG expression. More intense staining was detected in HPV16E5 expressing oral epithelial rafts for p-STAT3 and p-IRF3 than was detected in HPV6E5 or HPV16ΔE5 expressing rafts (Figure 3.5A). These differences were more subtle for ISG54 protein expression. OKF6 cells were transfected with HPV16WT, pCMV16E5, HPV16ΔE5, and pCMV6E5 in the presence or absence of NaB. Partially differentiated HPVE5 expressing OKF-6 cells that were not treated with NaB demonstrated markedly higher levels of ISG54 compared to cells that were terminally differentiated with NaB (Figure 3.5B). In partially differentiated cells, HPV16E5 mRNA induced ISG54 expression levels comparable to HPV16WT expressing cells. Statistically significant differences in ISG54 expression were detected in HPV16E5 expressing cells (10 fold) compared to cells transfected with HPV6E5 (p=0.007), and HPV16WT (5 fold) expressing cells compared to HPV16ΔE5 (p=0.007). In contrast, in NaB treated cells ISG54 levels were similar to vector control regardless of E5 expression and HPV16E5 and HPV6E5 demonstrated similar ISG activation activity. Of interest, albeit low relative to expression in the absence of NaB, those constructs that expressed other HPV oncoproteins, HPV16WT and HPV16ΔE5 expressed 2-3 fold higher levels of ISG54. HPV16WT was able to induce ISG54 expression but in the lower level comparing to non-NaB treated cells (3 fold to 8 fold, respectively). These results indicate that HPV16E5 induced interferon stimulated gene expression is cell differentiation dependent.
Figure 3.5 Upon differentiation E5 loses the ability to modulate the interferon response.

A) HR-HPV16E5 enhanced p-STAT3, p-IRF3 and ISG54 expression in raft culture (40X magnification). A) un-transfected control, B) HPV16WT, C) HPV16ΔE5, D) pCMV16E5HA, and E) pCMV6E5HA transfected cells. B) OKF6 cells treated with NaB were compared to OKF6 in normal media. The ISG54 gene fold changes were detected by real-time RT-PCR. Without NaB treatment, HPV16 and HPV16E5 were able to induced higher level of ISG54 comparing to HPV16ΔE5 and HPV6E5. HPV16E5 genome but not protein (pCMV16E5stop and HPV16E5stop) was induced ISG54 mRNA expression in comparable level to HPV16WT. Upon
the NaB induced terminal differentiation, HPV16 and HPV16E5 loss ISG54 induction ability. HPV16E5 and HPV6E5 showed no different on ISG54 induction.

Discussion

In this study we determined that HR-HPV E5 and LR-HPV E5 expression modulated oral epithelial cell differentiation. We show that expression of HR E5 enhanced the suprabasal state while suppressing terminal differentiation providing and optimal environment for oral HPV replication. Further, HR-HPVE5 increased expression of interferon stimulated genes in oral and primary keratinocytes undergoing chemically induced (sodium butyrate (NaB) and CaCl$_2$) or raft induced differentiation. While LR-HPVE5 also enhanced markers of suprabasal differentiation, terminal differentiation and interferon responses were not modulated similar to HR.

HPVs DNA replication is strongly tied to host cell differentiation. HR-HPVs reprogram cell cycle related epithelial differentiation to prolong viral replication (23). A previous study by Laimins group demonstrated that HPV31E5 supported cell proliferation and delayed cell cycle exit (39). HPV16E5 was reported to interrupted autophagic process in human keratinocytes by deregulated KGFR/FGFR2b expression on cell surface (40). In this study, HR- and LR-HPV E5 increased expression of spinous differentiation markers α-involucrin and K1 while K10 was expressed at higher levels under HR-HPVE5 expressing conditions than LR-HPVE5. We determined that HR-HPVE5 expressing cells, HPV16WT and HPV16E5, delayed terminal differentiation while cells expressing HPV6E5, HPV16ΔE5 and empty vector pCMVHA did not (Figure 3.4). Increased suprabasal differentiation and delayed terminal differentiation of oral epithelium enhanced the potential of HPV DNA replication.

HPV16E5 expression is largely controlled by a suprabasal cell regulated late viral promoter allowing for higher HPV16E5 expression levels in spinous cells than basal cells (41). HPVE5 modulates EGFR signal transduction (25, 26, 42) and EGFR expression in organotypic rafts is detected at higher levels suprabasal cells than in basal cells (31). Hence, enhanced E5
and EGFR expression would allow for increased suprabasal E5 mediated events. Upon calcium induced differentiation of primary cells, E5 mediated phosphorylation of both p38 and Erk1/2 was increased relative to undifferentiated cells. With differentiation, HR-HPVE5 was much more efficient at activation of these mitogen activated pathways than LR-HPVE5. In fact, LR-HPVE5 mediated Erk phosphorylation was detected at higher levels in undifferentiated cells. Our recent results in oral keratinocytes determined that HPV16E5 mediated interferon stimulated gene expression (ISGs) was both EGF and p38 dependent. This activity was specific to HR-HPVE5 as LR-HPV6E5 stimulated ISG levels comparable to empty vector (submitted). Here we show that HPV16E5 induced ISG expression was cell-differentiation dependent (Figure 3.5). Partially differentiated cells expressing HR-HPVE5 demonstrated higher ISG54 expression than HPV6E5 and HPV16ΔE5. It is thought that MDA5 is activated by dsRNA, such as poly(I):Poly(C), and viral mRNA may activate IFN expression through MDA5 (43). In differentiated organotypic oral epithelial rafts, E5 induced ISGs, p-STAT3, and p-IRF3. However, induction of terminal differentiation with NaB, resulted in a loss in the ability of HR-HPVE5 to induce ISG expression. These results suggested that HPV16E5 but not HPV6E5 induced ISG expression was differentiation dependent.

Type I IFN expression has been associated with selection of viral integrants. Differential gene expression studies detected a relationship between viral episomal loss and endogenous type I interferon inducible genes (44). β-interferon treatment of HPV31 infected human foreskin keratinocytes reduced episomal HPV but had no effect on viral integration (45). However, type 1 IFN treatment of W12 cells, and HPV16 episomal containing cell line, enhanced integration and E6 / E7 expression concurrent with reduction of episomal HPV genomes (46). Our studies suggest that HR-HPVE5 can mediate these interferon related events and are in agreement with previous studies where HPV16E5 was shown to induce β-interferon expression through IRF1 stimulation in HaCaT cells (33).
HPV E5 enhanced spinous differentiation and delayed terminal differentiation in oral epithelial cells providing an optimal environment for its proliferative activities and for viral replication. HR-HPV16E5 and LR-HPV6E5 demonstrated distinct phenotypes with differential activation of MAPK associated with cell differentiation. HR-HPVE5 mediated ISG expression was highly differentiation dependent. As interferon promotes integration, E5 mediated interferon responses may promote integration, complementing E6 and E7 in the genesis of cell transformation. Further studies of both HR- and LR-HPVE5 are required to understand the exact mechanisms of E5 in HPV associated lesions and malignancies.

Figure 3.6 HPVE5 related oral benign/malignancy model Cell proliferation and activation of intracellular immune responses are critical to the transformation of oral keratinocytes and subsequent cancer development. High risk HPV16E5 increased MAPK proliferation signals (p38, MEK1/2, and Erk1/2) and enhanced type I interferon responses (IFN promoter and ISGs) whereas low risk HPVE6E5 were comparable to vector control. HPV16E5 induced MAPK signaling and type I IFN responses are EGF- and cell differentiated-dependent manner.
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CHAPTER 4: GENERAL DISCUSSION

Human papillomaviruses cause both cervical and head and neck squamous cell carcinoma (HNSCCs) (1-4). Globally, these cancers have high mortality rates and excessive morbidity particularly in poor countries. Cervical carcinoma is the third most common cancer in women worldwide and second most common cancer in developing countries. Approximately 530,000 new cases occurred in 2008 and the mortality rate was about 52% (5, 6). HNSCCs have a similar mortality rate and are the sixth most prevalent human cancer worldwide with an estimated 405,000 new cases and 200,000 deaths each year (4, 7). In the United States, the incidence rate of HPV-negative HNSCCs has declined each year due to public health promotion of smoking cessation, however the incidence rate of HPV-positive HNSCCs continues to increase (8, 9). At least 30% of oropharyngeal squamous cell carcinomas (OSCCs) are HPV-associated with HPV16 accounting for 90% of the HNSCCs (3). High risk HPVs are highly associated with OSCCs but low risk HPVs are rarely found in carcinomas (3, 10). Low risk HPVs, such as type 6 and 11 are dominant types in oral warts and recurrent respiratory papillomas (11-13). Mixed type of high risk and low risk HPVs are co-localized in oral cavity and both expressed in oral hyperplastic lesions (14). Our group previously detected 35 distinct oncogenic and low risk HPV types including type 6, 11, 16, and 18 in oral fluids and tissues (15). Although high risk and low risk HPVs are detected in both cervical and oral regions, the environments are distinct and HPVs pathogenesis may be different. The exact molecular mechanisms of HPV-induced OSCCs formation is not completely clear but has been tied to chromosomal abnormalities mediated by HPV oncogenes. E5, E6, and E7 are three oncogenes found in high risk HPV. The functions of these three oncogenes have been extensively
investigated in the context of cervical cancer, but their roles in HPVs-positive OSCCs pathogenesis needs to be further studied. While E6 and E7 have been proven to induce cell transformation in oral keratinocytes (16-18), the role of E5 in oral epithelial cells remains poorly understood and has not been previously investigated.

The studies presented in this thesis characterized HPV- related oral pathogenesis associated with oncogenic high risk HPVE5 and low risk HPVE5 in primary and immortalized oral epithelial cells, in monolayers and in rafts. This thesis was focused on understanding how HPV modulates oral immune responses in vivo and in vitro. Specifically, the role of HPVE5 in the modulation of oral keratinocyte immune responses was determined. High risk HPV16E5 and low risk HPV6E5 were compared to identify the different molecular mechanisms potentially underlying HPV-related OSCCs and HPV-related oral warts.

High risk HPV16E5 and low risk HPV6E5 share some similarities. They are hydrophobic trans-membrane proteins found mainly in the endoplasmic reticulum and golgi apparatus (19, 20). Both prolong cell surface EGFR expression and are believed to use this mechanism to transform murine keratinocytes (21-24). However, the mitogenic induction of human keratinocytes was only assessed for high risk HPVE5 (24-26). Unlike E6 and E7 whose structural proteins and mechanistic functions on the interruption of normal cell cycle have been well compared between high risk HPVs and low risk HPVs (27), there is only one study comparing high risk HPVE5 and low risk HPVE5 protein structures, and no studies, to our knowledge, that directly compare activity/ and mechanisms (19).

We first sought to determine the relationship between HIV, oral HPV and oral immune modulators. Our study in the appendix discerns the prevalence of oral HPV in an HIV infected cohort. This study was part of the HRSA funded UNC HIV Oral Health Demonstration Project that provided comprehensive dental care. Clinical periodontal measures, saliva, throat washes, GCF, and plasma were collected from HIV-positive and HIV-negative controls at 0, 12 and 24 months. Markers of local immunity (SLPI, oral IgA), and of immune activation (sCD14, IL-6,
CD163, CXCL-10), bacterial loads, HPV- and HIV- viral loads were assessed. Oral HIV viral loads were correlated with systemic HIV viral loads. HIV-patients who maintained low level HIV viral loads but had no improvement of CD4 counts even after HAART demonstrated increased systemic CD4 counts after dental care treatment. Reduced oral pro-inflammatory cytokines were related to the improvement of periodontal diseases. After periodontal treatment, patients experienced less cytokines expression concurrent with the shifting of periodontal diseases to less severity. Interestingly, the reduced plasma levels of pro-inflammatory cytokines were related to cytokine levels in oral fluid. The data suggested that oral inflammation could be a source for systemic inflammation. Microbiome translocation to intestinal area may interrupt CD4 generation in local lymph node thereby attribute to severe chronic conditions in HIV infected patients (28). Improved CD4 counts and reduced local and systemic inflammatory markers highlight the importance of dental intervention for the improvement of oral and systemic health of HIV- infected patients.

HIV-infected patients are at risk for HPV infection (29) and are at risk for the development of HPV-related head and neck cancer (29). The prevalence of HPV DNA in the oral cavity is high in HIV-infected patients with low CD4 counts (30) and oral warts are prominent oral lesions in HIV patients on HAART (12, 13). In the study presented here, those individuals who were HPV-positive exhibited higher oral cytokine expression compared to HPV-negative individuals. Soluble-CD14 was expressed at statistically higher levels in HIV-positive oral/ HPV positive individuals compared to HIV-positive oral HPV-negative subjects. These data imply that HPV plays a role in modulating local immune responses in the oral cavity. We further investigated if HPVE5 alters the function of innate immune responses in oral keratinocytes.

While HPVE5 is a known oncoprotein, few studies on E5 have been performed and no studies have been performed in oral epithelial cells. Chapter 2 (AIM1) determined that high risk HPVE5 mediated MAPK signaling pathway as a means to activate type I-IFN responses in oral epithelial keratinocytes. Differential activation of type I IFN promoter activity, type I stimulated
gene expression, and IFN-related proteins including STAT1, STAT3, and IRF3 were detected in association with high risk but not low risk HPVE5. We have determined the E5 molecule regulated different signal transduction pathways in oral epithelial cells than those activated by E5 in previously published studies of cervical cells. Both the cancer-associated high risk E5 protein and high risk E5 mRNA activated the MAPK induced IFN response. These responses were both EGF and differentiation-dependent. Low risk viruses important to wart development demonstrated a significantly different profile. Low risk HPVE5 was most active in undifferentiated cells while high risk viruses flourished in partially differentiated cells.

First, signal transduction was compared between high risk HPV16E5 and low risk HPV6E5. Our data demonstrated that different pathways were used by high risk and low risk HPVs in oral keratinocytes. High risk and low risk HPVE5 were previously shown to induce MAPK signaling by interrupting the internalization and degradation of EGFR to result in epithelial cell proliferation and overgrowth (31). In contrast to the high expression of p-Akt and p-Erk1/2 mediated by HPV16E5 in cervical cancer cells (32), our data of HPV16E5 expressing oral cells detected enhanced expression of p-MEK/Erk 1/2 and p-p38 but not p-Akt. Further, high p-Akt expression was detected in low risk HPV6E5 expressing cells. Cox2 inhibition of p-p38 induction in HPV16WT and HPV16E5 expressing cells, but not in low risk HPV6E5, confirmed the differential use of the p38 pathway by the E5 proteins. Deletion of the E5 ORF from the HPV16 genome resulted in MAPK modulation comparable to low risk HPV6E5 expressing cells. JNK expression was unaffected by E5. HPV16E5 mRNA alone was able to induce MAPK signaling in partially differentiated oral keratinocytes.

Second, we investigated the role of EGF on HPVE5. High risk HPV16E5 was unable to induce p-Erk1/2 in the absence of EGF. In the absence of EGF, high risk HPV16E5 behaved similar to low risk HPV6E5. In this study, HPVE5 signaling has been shown to consistently modulate the interferon response in oral epithelial cells. IFN promoter activity and IFN-stimulated genes were modulated by high risk HPVE5, p38 mediated and were ablated by Cox-
2 inhibition. HPV16E5 mRNA alone was able to induce ISGs in partially differentiated oral keratinocytes. Our results were consistent with p-p38 induced ISRE activities in mice hematopoietic progenitor cells (33). We have shown that HPV16E5 uses the MAPK signaling pathways to induce the expression of ISGs. Similar to MAPK expression, ISG expression was diminished in the absence of EGF. Addition of EGF promoted HPV16E5 modulation of IFN responses. Interestingly IFN, a therapeutic drug sometimes used to treat viral infection, has been shown by others to increase the possibility of HPV integration (34). The concept of integration selection by IFN treatment has been suggested by the Stanley group previously (35). Interferon beta treatment in the naturally HPV16-infected cervical cell line W12 promoted the reduction of episomal viruses concurrent with an increasing in integrated viruses with high E6 and E7 expression (34, 36). Not only the selective effects on integration, but prolonged treatment of IFN-gamma has also been shown to induced de novo viral integration in HPV16 transfected keratinocytes (37). Integration leads to the loss of viral episomes and of HPVE2 which acts as a suppressive molecule to control the expression of E6 and E7 in turn suppressing E6/E7 associated higher incidence of chromosomal abnormalities (35).

Both STAT1 and STAT3 were enhanced by HPV16E5 but not HPV6E5, as was also shown in our study. This finding is important since STAT3 expression is associated with head and neck carcinogenesis and tumor progression (38) and STAT3 expression has previously been linked to p-p8 expression levels (39). HPV16E5 mediated signal transduction in chapter 2 was linked to p-p38, Type 1 interferon response, and to STAT3 expression. In the contrary, low risk HPVE5 showed minimal effects on p-p38 and STAT3. In the context of the whole HPV genome, E5 modulation of IFN responses was overshadowed by the suppressive effects of E6 and E7.

Of potential importance to oncogenesis, was our discovery that high risk HPVE5 appeared to control the expression of splicing E6 in our system. When E5 was present, E6 remained unspliced and E7 levels were lower, however the deletion of E5 resulted in splicing of
E6 and up-regulation of E7. HPVE6 is a protein critical to oncogenesis that deregulates the cell cycle and apoptosis. We have shown for the first time that HPVE5 expression may modulate expression of high risk HPVE6. We have shown that E5 inhibits E6 splicing which does not allow expression of the truncated protein called E6*. E6* was expressed in oral epithelial cells containing viruses deleted for E5 (HPV16ΔE5) and HPV16E5stopcodon but not in the HPV16WT expressing cells. E6* was related to EGF presence in cervical cells (40) but has never been reported in oral keratinocytes, and has not previously been linked to E5. E6* has been shown to increase E7 transcription (40). Given our results, E5 may indirectly control E7 oncogene expression. Future studies will further determine the role of E5 on HPVE6 and E7 gene expression.

In the context of natural infection, a balance between HPVE6/E7 IFN suppression and HPVE5 activation of innate immune responses must be achieved. The findings presented in this thesis suggest the importance of temporal expression of high risk HPVE5 in the establishment of cancer. Early in cancer development, HPV16E5 mediated immune responses that may modulate integration, and modulate E6/E7 gene expression. Post integration, E5 expression may be lost, resulting in uncontrolled expression of E6 and E7 with subsequent oral epithelial cancer progression. Future studies are ongoing to assess the E5-integration link.

The HPV life cycle is tied to epithelial cell differentiation. HPV episomes are highly amplified at supra-basal epithelial cell layers prior to formation of complete virions at cornified layers. At basal cell layers HPVs maintain low copy numbers with about 50-100 copy numbers per cell (2). Prolonged epithelial differentiation could benefit viral replication and amplification thus is advantageous for viruses to interrupt and delay terminal differentiation and apoptosis. HPV16E6 and E7 have been shown to alter cell differentiation in human foreskin keratinocytes (41) and one study showed that HPV16E5 mutants did not alter keratinocyte differentiation and did not affect the formation of virus like particles (42). The role of HPVE5 on cell differentiation had not been reported in the context of oral keratinocytes prior to work reported here.
In chapter 3 (AIM2) it was determined that several epithelial host factors influence HPVE5 mediated innate immune responses. High risk HPVE5 induced hyper-proliferation, enhanced spinous differentiation but delayed terminal differentiation. High risk HPVE5 was assessed individually and in the context of the HPV16 genome in primary oral keratinocytes and in immortalized oral keratinocytes that were established in both organotypic raft culture and monolayer culture. In organotypic raft culture, both high risk and low risk HPVE5 enhanced hyper-proliferation in oral keratinocytes compared to non-viral infected oral keratinocytes. Koilocyte formation is characterized by acenetric, hyper-chromatic nuclei displaced by large perinuclear vacuole. HPVE5 transfected cells exhibited koilocytes, a manifestation unique to HPV infection as previously described in other studies (43). The appearance of koilocytes confirmed the successful culture of E5 expressing primary foreskin keratinocytes and immortalized oral keratinocytes in our system. Both high risk and low risk HPVE5 activated spinous differentiation. K1mRNA, K10 protein, and involucrin protein in transfected raft cultures. High risk HPVE5 induced higher levels of K10 and HPV16ΔE5 demonstrated loss of K1, K10 and involucrin expression compared to HPV16WT. HPV16WT and HPV16E5 delayed terminal differentiation in oral keratinocyte cell lines. Our results support previous data indicating a role for high risk HPVE5 in cell proliferation and delayed cell cycle exit (44). Importantly, HPV16WT and HPV16E5 expressing cells exhibited the delayed terminal differentiation while low risk HPV6E5 and HPV16ΔE5 did not. Moreover, in the monolayer culture system using primary human keratinocytes HPVE5 induced MAPK expression in cell differentiation dependent manner. Lower p-Erk1/2 and p-p38 induction was detected in undifferentiated primary HFK cells compared to differentiated cells, while the low risk HPV6E5 showed enhanced p-Erk1/2 activity in undifferentiated cells. There were two scenarios where high risk HPV16E5 behaved similar to low risk HPV6E5, in the absence of EGF and during terminal differentiation. High risk HPVE5 consistently impeded terminal differentiation. The delayed terminal differentiation and induced cell proliferation may allow for enhanced HPV DNA replication. These results suggest a role for
high risk HPVE5, but not low risk HPVE5, to enhance the partially differentiated state allowing for increased E6 and E7 expression that may facilitate transformation.

During terminal differentiation, ISG expression was lost. Both EGF and partial differentiation supported high risk HPVE5 modulation of IFN responses. In the presence of EGF, E5 mRNA alone was able to induce ISGs expression at levels comparable to HPV16WT. It is possible that E5 mRNA, in form of double stranded RNA, may trigger MDA5 or RIG-I like receptor to induce interferon and interferon-stimulated genes responses. MDA5 and RIG-I are cytoplasmic receptors that sense long and short intracellular ds-RNA, respectively (45, 46).

During normal transcription, HPVE5 mRNA and protein both induced type I IFN responses. Interestingly, HPV16WT and high risk HPV16E5 induced higher levels of STAT3 protein expression compared to HPVΔE5 and low risk HPV6E5 in organotypic cultures. Overexpression of STAT3 is strongly correlated with head and neck cancer development and progression (47). The persistent activation of STAT3 may interrupt the normal regulation of anti-apoptotic genes; Bcl-XI, Mcl-1, Bcl-2, and of proliferation related genes; Myc and cyclinD1 (48). The expression of STAT3 is related to p38 activation in head and neck cancer (39). High risk HPV16E5 and low risk HPV6E5 demonstrated differential activation on p-p38 in oral keratinocytes. This was demonstrated by data from both chapters 2 and 3. The p-p38 inhibition in HPV16E5 expressing cells confirmed the importance of p-p38 on STAT3 expression. STAT3 protein was reduced after p-p38 suppression. Hence, high risk HPV16E5 used p-p38 pathway to induce STAT3 expression in oral keratinocyte.

High risk HPVE5 may be a key factor in oropharyngeal carcinogenesis. Concurrent with the distinct functions of high risk HPV16E5 to enhance spinous differentiation and delay terminal differentiation, high risk HPVE5 provide optimal conditions for HPV replication while inducing STAT3 to prolong cell proliferation (our study, (49)). Moreover, HPVE5 controls the expression of other oncoproteins E6 and E7 through its ability to modulate differential splicing. Ongoing
studies suggest that this may occur due to E5’s ability to modulate the splicing factor hnRNPA1 (data not shown).

In conclusion, a model is proposed where HPVE5 may regulate oral benign/malignant lesion development based on the level of cellular differentiation (Figure 4). In the basal layer, low risk HPV E5 is highly proliferative while in the supra-basal/partially differentiated layers low risk is less active and high risk HPVE5 activates proliferative pathways and modulates innate immune responses. Cell proliferation and activation of intracellular immune responses are critical to the transformation of oral keratinocytes and subsequent cancer development. High risk HPVE5 increased MAPK proliferation signals (p38, MEK1/2, and Erk1/2), STAT1, STAT3, and enhanced type I interferon responses (IFN promoter and ISGs); both were dependent on both EGF and cell differentiation. Low risk HPVE5 did not modulate these processes. High risk HPVE5 inhibited terminal differentiation and HPVE5 was not active within the most terminally differentiated oral cells. Additionally, HPV16E5 played a role in the regulation of E6 splicing. In summary, high risk HPV16E5 may be a key facilitator of oncogenic progression in the oropharyngeal region while low risk HPV6E5 exhibited proliferative capability only in oral basal cells. High risk HPVE5 may be a potential therapeutic target for HPV-related oropharynx cancers.
Future directions

Future studies are required to tease out the role of E5 in head and neck tumor formation and tumor progression. Since we have determined that E5 modulates interferon responses, it will be important to determine whether HPVE5 induced IFN responses facilitate integration selection in oral epithelial cells. If so, what is required for HPVE5 induced HPV integration? It would also be interesting to determine how HPVE5 DNA and mRNA modulate type I IFN responses. Does HPV nucleic acid trigger RIG-I, MDA-5 receptor or any other specific PAMP sensors? The intracellular mechanisms of HPV modulated innate immune responses remains unclear and need to be further investigated.

We have determined that E5 modulates the expression of p38 and the splicing of E6/E7. Does HPVE5 control the translocation of hnRNPA1, the splicing regulator, through the modulation of p-p38? Does HPVE5 utilize splicing machinery to regulate E6 and E7 expression or potentially the expression of L1? Studies are underway to determine if this is true. As E5
mediated pathways are deciphered in oral epithelia, long-term studies could target E5 mediated cellular pathways to diminish HPV associated oral diseases.
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APPENDIX: PERIODONTAL TREATMENT IMPROVES IMMUNE ACTIVATION/INFLAMMATION IN HIV-POSITIVE PATIENTS.

Abstract

Highly active antiretroviral therapy (HAART) improves HIV survival; however patients still suffer from adverse events and mortality. Persistent oral bacterial infection may induce abnormal systemic immune activation and inflammation, major causes of morbidity and mortality in the setting of HIV. We hypothesized that periodontal intervention would improve immune activation and inflammation in HIV-patients. In the IRB approved UNC HIV Demonstration project clinical periodontal measures, saliva, throat washes, gingival crevicular fluid (GCF), and plasma were collected from HIV positive (n=196) and HIV negative controls (n=10) at 0, 12 and 24 months. Groups were stratified based on time of HIV diagnosis and time out of dental care. HIV patients were provided comprehensive dental care and OHI. Markers of local immunity (SLPI, IgA) and of immune activation (sCD14, IL-6, CD163, CXCL-10) were assessed by ELISA. The correlation of markers in oral and plasma samples was determined. Bacterial load was assessed by 16sDNA qPCR. sCD14, IL-6 and CXCL-10 were negatively correlated with bacterial copy number (r= -0.775, -0.358, -0.446, respectively). Oral and plasma viral loads were positively correlated (r=0.625) and as were sCD163 levels in GCF and plasma (r=0.341). HAART subjects demonstrated diminished oral inflammation. Oral SLPI and sIgA levels were higher in ART suppressed patients compared to those with HIV viral load (VL)>50 (p<.05). sCD163, IL-6 and CXCL-10 were significantly higher in suppressed individuals and decreased in both the plasma and oral compartments with dental treatment (p<.05). Subjects with HIV VL (<50 copies/cell) and CD4 (<350 cells/ml) at baseline demonstrated marked decreases in clinical oral inflammation, local and systemic inflammatory markers (p-values; BI=0.019, GI=0.043, sCD163=0.001, IL-6=0.001, sCD14=0.028, CXCL-10=0.055) and a mean CD4 improvement of 152 cells/ml after dental care. In conclusion, improved CD4+ T cell counts and reduced local and
systemic immune/inflammatory markers highlight the importance of dental intervention to the systemic health of the HIV-infected patients.

Introduction

Human Immunodeficiency virus (HIV) infection/AIDS is a global pandemic. Since recognition of the virus 30 years ago, HIV has resulted in more than 30 million deaths. According to UNAIDS report, there were approximately 6 million people living with HIVs and 240,000 deaths in 2012 (1). Although the numbers of new cases has been reduced due to the massive effort to provide Highly active antiretroviral therapy (HAART) treatment and HIV prevention programs, HIV infected people still suffer from the non-AIDS conditions which shorten their lives compared to HIV-negative patients (2-6). The HIV associated non-AIDS-defining (HANA) conditions are caused by the abnormal persistent inflammation and hyper-immune responses despite of the effect of HAART and the suppressive HIV viral load. The common features of HANA composed of multiple organ complication and organ failure including cardiovascular disease, liver disease, renal dysfunction, non-AIDS defining cancer, neurocognitive decline, weakness, weight loss and increasing risks of disability (7). Increasing systemic inflammatory markers has been report to be associated with non-AIDS conditions and death (8, 9). The possible major cause of HANA is the impaired gut T cells and the translocation of gastrointestinal microbiome and their products, such as bacterial lipopolysaccharides (LPS), through the intestinal epithelial barrier and lamina propria to local lymph nodes and extra intestinal sites (10). Taking into account that oral infection has been purposed to be a possible source of systemic inflammation by transient bacteremia of oral pathogens at extra-oral sites (11). Since chronic oral inflammation including gingivitis and periodontal diseases are common in HIV patients (12). Periodontal diseases may lead to abnormal systemic immune activation and inflammation, major causes of morbidity and mortality in the setting of HIV.
Periodontal disease is a chronic inflammatory disease of teeth supporting tissues including gingiva, alveolar bone, periodontal ligament, and collagen around teeth caused by the pathogenic microflora and the interaction between microflora and host immune responses. Oral microorganisms are capable to activate inflammatory cells including polymorphonuclear cells, lymphocytes and macrophages to produce pro-inflammatory mediators and subsequently destroy periodontal tissue (13-15). Bacterial pathogens stay in periodontal area as a complex community (14, 16). Dental plaque attached to tooth surface as a biofilm composed of the co-localization of facultative gram positive bacteria, such as Streptococcus sanguis, Streptococcus mitis, Actinomyces naeslundii, Eubacterium species, and facultative gram negative bacteria, such as Porphyromonas gingivalis, Prevotella intermedia, Fusobacterium nucleatum, and Bacteriodes forsythus. Peridontal bacteria induce periodontal diseases either by directly contact of bacteria pathogenic associated molecular patterns (PAMPs) to host pathogen-associated pattern recognition receptors (PRRs) or indirectly activate peripheral blood monocyte cells. The pathogenesis of periodontal diseases in HIV infected patients is the same as in healthy individual but exhibits more severity due to HIV suppress immune responses and the oral pathogen induced HIV reactivation (17). Pathogenic bacteria PAMPs directly contact to PRRs, thus further activate the expression of pro-inflammatory cytokines and chemokines for innate and adaptive immune activation (18-20). Periodontal is now considered as bacterial-virus infection (21). The detectable level of Herpesviruses including Ebstein-Bar and cytomegalovirus were presented as a higher prevalence in patients with chronic periodontitis compared to non-periodontitis (22). HIV1 infection also showed the association with the prevalence of chronic periodontitis (17). Study by Kroidl et al. demonstrated about 70-80% of HIV infected patients with chronic periodontitis (23). HIV patients under antiviral therapy (ART) demonstrated the increasing level of CD4 counts together with reduced number of HIV viral load after periodontal treatment (24). Moreover, Porphyromonas gingivalis and Fusobacterium nucleatum have been shown to promote the reactivation of HIV (17, 25-27). The mechanisms of viruses induce
Periodontitis is still not well defined. Viruses may impair local host immunity and mediate local inflammatory cytokines responses thus increase the virulence of periodontal pathogens and severity of periodontal tissue destruction.

The detection of extra-oral sites of oral microbiome supports the concept of the oral microbial pathogenic burden contributes to systemic immune activation (11). Oral microbial pathogens have been detected in systemic infection as seen in atherosclerosis (28-30) and constitutively showed the association with preterm birth, stillbirth, and early onset neonatal sepsis (31-33). The possible source of chronic systemic inflammation in HIV patients is the translocated oral microbial or microbial products from oral cavity into gut. Impaired CD4 T-cells in the HIV infected patients could not inhibit the translocation of gut microbial into extra-intestinal tissues (10) then allowed for chronic hyper-immune responses. Brenchly et al. also suggested that microbial translocation could induce mucosal immune dysfunction and disease progression in HIV infected patients (34). Since dental care treatment can solve the inflammation and microbial infection in oral cavity, we sought to find the effects of periodontal treatment on local oral and systemic inflammation in HIV-seropositive patients by the investigation of systemic inflammation, monocyte/macrophage activation, and HIV activation markers in the oral fluid and plasma. We also investigated the co-infection and the role of HPV on immune modulation in oral cavity in HIV infected patients. Since HPV is one of the pathogenic microbial found in oral fluid of HIV positive patients (35, 36), oral HPV infection is potentially related to chronic systemic inflammation.

In this study, we showed that periodontal intervention improved immune activation in HIV infected patients. Periodontal treatment contributes to reduced levels of both local and systemic pro-inflammatory cytokines expression including secretory leukocyte peptidase inhibitor (SLPI), secretory immunoglobulin A (sIgA), sCD14, IL-6, sCD163, and CXCL10. Meanwhile, patients who received periodontal treatment experiences increased CD4 counts despite the suppressive level of HIV viral load. We also found the localization of oral HPV in HIV infected patients.
Diminishing oral inflammation may reduce risk of chronic inflammation, therefore, dental care treatment and oral health promotion is an essential care for HIV infected patients.

**Materials and methods**

**Patient selection and samples:** this study is part of Oral HIV and AIDS Research Alliance (OHARA) at University of North Carolina at Chapel Hill. In the IRB approved UNC HIV Demonstration project, a total of 196 HIV seropositive patients participated in the project. Samples from a total of 129 patients were randomly selected for our study. The study groups had been stratified into three groups based on time of HIV diagnosis and time of dental care. Group 1 (N=42) was patients with the newly HIV infection diagnosed in the last 12 months and had no dental care treatment in the last 12 months. Group 2 and 3 were the patients who had been diagnosed with HIV infection during 1985 through 2007. Group 2 (N=51) was patients who had been diagnosed with HIV infection and had no dental care treatment for the last 12 months. Group 3 (N=36) was patients with HIV infection diagnosed and had dental care treatment prior to the study. Periodontal measures (periodontal pocket depth, attachment loss, bleeding on probing, gingival index), saliva, throat wash, gingival crevicular fluid (GCF), and plasma were collected at baseline (first entry, no treatment), 12 months, and 24 months. Peridontal status was classified into mild, moderate, and severe following American Academy of Periodontology (AAP/CDC) classification (37). Healthy people (N=10) were also recruited to the study as a control. GCF, saliva, and blood from healthy control were collected for 3 times points at one month apart from each time point. HIV patients were provided oral hygiene instruction and comprehensive dental care including scaling and root planing, extraction, filling, root canal treatment, and/or denture if needed. The maintenance visits were every 6 months after the first entry. CD4 counts, oral bacteria load, oral and plasma HIV viral load were also measured. To analyze periodontal diseases based on clinical, microbial, inflammatory, and host response data, we also used Biofilm-Gingival Interface (BGI) index to classify periodontal severity (38).
Sample collection: Patients were asked to gargle with 5 ml of 0.9% normal saline for throat wash collection followed by 5 minutes of saliva collection. About 5 ml of un-stimulated whole saliva was collected into 50 ml centrifuge tube. After collection, saliva was centrifuged at 20000 G for 1 minute and only supernatants were collected for study. GCF was collected by using paper point inserted into gingival pocket at mesio-buccal region of first molar for all four first molars. If first molar did not exist, premolar was used instead. GCF was dissolve with 200 microliter calibrate diluent before analysis. Throat wash was centrifuged at 3000 G for 10 minute to separate throat wash pallets from supernatant.

Enzyme-linked immunosorbent assay (ELISA) for immune mediators: ELISA assay was used to measured level of immune mediators; IL-6 and sCD14, SLPI, interferon-inducible protein CXCL-10 (Quantikine Kit; R&D systems), IgA (Creative Diagnostics), and sCD163 (IQ Product; Trillium Diagnostic, LLC). Calibrator diluent was used to dilute samples as following dilution factor: Saliva; 1:500 (SLPI), 1:10 (sCD14 and IL-6), 1:1 (CXCL10 and sCD163), 1:2000 (IgA); throat wash; 1:200 (SLPI), 1:1 (sCD14, CXCL10, and sCD163), 1:500 (IgA); GCF 1:1 (SLPI), no dilute (sCD14, sCD163, and IL-6); plasma 1:200 (sCD14), 1:10 (CXCL10), 1:500 (sCD163), and 1:5 (IL-6). Diluted samples were assayed in duplicate followed manufacturer’s instructions. Concentrations of mediators were determined by optical density at manufacturer’s recommend wavelength using a microplate reader (Epoch microplate spectrophotometer; Biotek). The duplicate readings were averaged and the values were multiplied by the dilution factor.

Quantitative real time polymerase chain reaction (q-PCR) for oral bacteria and oral HPV load: Throat wash pallet DNAs collected at baseline, 12 months, and 24 months from 45 patients were assessed by q-PCR using 16sDNA primer pairs for oral bacterial loads as followed U16S119OR 5’-CTCACGRCACGAGCTGACGAC-3’ and U16S1020F 5’-TTAAACTCAAAGGAAATGACGG-3’. HPVE1 primer pairs were used to detect oral HPV loads
as followed HPVE1F1 5’-ANANGCTGTGCAKGNCTAAAACGAAG-3’ and HPVE1R1 5’-AGTTTCCACTTCAGTATTGCCATA-3’.

**Statistic analysis**: Immune mediators, SLPI, sIgA, CXCL-10 from saliva, throat wash, GCF and plasma were compared between group (group 1, group 2, group 3, and control) and time points (baseline, 12 months, 24 months). CD4 counts, oral bacterial load, oral and plasma HIV viral load, and periodontal parameters were also compared. Kolmogorov-Smirnov test was used for normality test. ANOVA and Mann-Whitney were used for statistic difference analysis between groups. Pearson and Spearman test were used to analyze the correlation between oral and plasma HIV viral load, oral and plasma immune mediators. The threshold of statistic significant was at P-value <0.05.
Results

Plasma viral load, CD4 counts, periodontal status and immune mediators improved over time with periodontal treatment. HIV positive subjects (N=129) received prophylaxis or debridement every 6 months intervals over the course for 24 months. The demographic data and baseline periodontal measurements of all HIV infected participants are shown in Table 1.

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| On ARV at baseline | 80% |

| Clinical parameters (mean±SD) | Mean VL  | 18244±42095 |
|                              | Mean CD4  | 569.4±308.6 |
|                              | Mean extent GI | 81.82±28.72 |
|                              | Mean extent BOP | 66.67±24.42 |
|                              | Mean extent PD | 34.10±23.09 |
|                              | Mean extent AL | 14.28±18.20 |

Table 1. Demographic data at baseline. IVDU=intravenous drug use, MSM=men who have sex with men, VL=viral load, GI=gingival index, BOP=bleeding on probing, PD=pocket depth, AL=attachment loss. ARV=anti retroviral drug therapy.
Periodontal parameters were improved within 24 months after periodontal treatment compared to baseline (figure 1A). The extent pocket depth≥4 mm with bleeding on probing and extent attachment level≥4 mm with bleeding on probing significantly reduced at 24 months (P<0.0001 and P=0.013, respectively) (figure 1A). Periodontal treatment also showed the effects on reduced plasma viral loads and increased CD4 counts within 24 months. Plasma HIV viral load and CD4 counts improved from 569.4±308.6 to 752.7±372.3 (all time points) (Figure 1B and C). The markers of immune and inflammatory activation were higher in HIV-infected patients compared to healthy control and were significantly reduced after periodontal treatment (throat wash sCD14: P=0.003; GCF sCD163 P=0.035; saliva sCD163 P=0.038, saliva IL-6 P=0.03) (Figure 1C).
Figure 1. Plasma viral load, CD4 counts and periodontal status improved over time with periodontal treatment and oral markers of immune activation, IL-6 and sCD163 decreased. HIV positive subjects (N=129) received prophylactic debridement at 6 months intervals over the course of 24 months. A) periodontal measures, B) plasma viral load and CD4 counts improved from 569.4±308.6 to 752.7±372.3 (all time points), and C) CD4 counts and inflammatory markers in patients with low CD4 counts at baseline.

We also used BGI index to classify periodontal status in HIV patients. The benefit of BGI index is the adding value of bleeding on probing (BOP) scores to the value of pocket depth (PD) scores (38). Here we showed the relationship between periodontal severity (BGI index) to pro-inflammatory cytokine expression, to CD4 counts and, to plasma HIV viral loads. Level of pro-inflammatory cytokines (IL-6 and sCD14) in P2 (PD≥4 mm with moderate BOP), and P3 (PD ≥4 mm with severe BOP) were reduced after 12 and 24 months of first periodontal treatment (Figure 2A-B). Interestingly, HIV viral loads were highly expressed in P3 patients and reduced over time of treatment (Figure 2C), concurrent with the increasing of CD4 counts (Figure 2D). In the contrary, the gingivitis (PD≤3 mm with mild BOP) and P2 groups demonstrated low HIV viral loads. Patients in gingivitis groups were the best whose CD4 counts were improved over the treatment. The periodontal-cytokine-microbial expression from this study emphasized the important of periodontal treatment to the improvement of viral control in HIV infected patients.
Figure 2. The shifting of periodontal inflammation, viral loads, and CD4 counts after periodontal treatment. Periodontal severity was classified by BGI index into healthy, gingivitis, P2, and P3. Periodontal treatment improved A) IL-6 expression (pg/mL), B) sCD14 expression (pg/mL), C) plasma HIV viral load (copies number/cell), and D) CD4 counts (cell/mL).
The oral sCD14, IL-6 and CXCL-10 was negatively correlated with the copies number of oral bacterial 16SDNA (Spearman r= -0.775, -0.358, -0.446, respectively). Oral viral load showed the positive correlation to plasma viral load (Spearman r=0.625) and as were GCF and plasma samples (sCD163, Spearman r=0.3741). CD4 counts showed the negative correlation to plasma viral load (Spearman r=0.2623) (Table 2).

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<tr>
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Table 2. Comparison of the oral and blood compartments.

CD4 counts, periodontal status, and oral immune mediators improved over time with periodontal treatment in a subset of HIV patients who had longstanding HIV infection and had no dental care for 12 months. Group 2 patients (N=51), HIV patients who were diagnosed as HIV infection for longstanding but had no dental care treatment for at least 12 months before study entry, showed the improved periodontal parameters at 12- and 24-months after first periodontal treatment. The extent pocket depth $\geq 4$ mm with bleeding on probing and the extent attachment level $\geq 4$ mm with bleeding on probing tend to decline, meanwhile, bleeding on probing and extent gingival index significantly reduced after received periodontal treatment (P<0.05) (Figure 3A). Levels of periodontal parameters did not related to plasma HIV load. Both HIV suppressed (HIV viral load<50 copies/cell) and not suppressed (HIV viral load $\geq 50$ copies/cell) showed periodontal improvement.

Mean CD4 count in this group improved from 553.1±318.7 to 813.3±486.7 within 12 months of periodontal treatment. Levels of oral inflammatory markers saliva sCD163 and IL-6 were also significantly reduced (Figure 3B and 3C).
Figure 3. CD4 counts, periodontal status, and oral markers of immune activation, IL-6 and sCD163 improved over time with periodontal treatment in a subset of HIV patients who longstanding HIV infection and had been out of dental care for over 1 year (n=51). Improvement was seen. A) periodontal measures both in suppressed (HIV viral load<50 copies/cell) and those who were not suppressed (HIV viral load≥50 copies/cell). B) CD4 counts improve from 553.1±318.7 to 813.3±486.7 in this group, and C) improved inflammatory markers sCD163 and IL-6.
Local and systemic measures of immune activation, CD4 counts, and periodontal status improved over time with periodontal treatment in a subset of HIV suppressed but low CD4 counts patients. Subset of HIV infected patients in group 2 who had longstanding diagnosed as HIV infection and were under condition of HAART with suppressed plasma HIV viral load (<50 copies/cell) but had no improvement of CD4 counts (<350 cells/ml) (n=7) and had no dental treatment for over 12 months at study entry were selected for immune mediator analysis. Interestingly, mean CD4 counts rose from 194±112.7 to 353.8±213.9 (mean gain=152 cell/mL) within 24 months (Figure 4A). Periodontal parameters of patients in this subset showed the improvement at 12 and 24 months (Figure 4B). Local measures of inflammation sIgA and SLPI diminished to the comparable level to healthy controls (Figure 4C). The immune mediators were improved in both oral (saliva) and plasma markers. Both local oral and systemic plasma markers of immune mediators (sCD14, sCD163, IL-6, and CXCL-10) were significantly reduced after periodontal treatment. At 24 months, levels of sCD14, sCD163, IL-6, and CXCL-10 were reduced to the comparable level to healthy control (Figure 4D).
Figure 4. Local and systemic measures of immune activation, CD4 counts and periodontal status improved over time after periodontal treatment in a subset of HIV patients who were ART suppressed (HIV viral load < 50 copies/cell) and had low CD4
counts (<400 cells/ml) and had been out of dental care for over 1 year (n=7). A) Mean CD4 counts rose from 194±112.7 to 353.8±213.9. B) Periodontal measures (extent PD, AL, BOP and GI) improved significantly (p<.005). C) Local measures of inflammation sIgA and SLPI diminished to levels comparable to healthy controls. D) Both local oral and systemic plasma markers of immune activation (sCD14, sCD163, IL-6, CXCL-10) were significantly reduced after periodontal treatment.

**Human papillomaviruses DNAs was detected in oral fluid of HIV infected patients.** By using q-PCR with HPVE1 primers, HPVs DNAs were detected in throat wash pallets in 32% of HIV infected patients (N=28) (Figure 5A). We compared the levels of immune mediators between HPV positive and HPV negative and found the significantly lower levels of sCD14 (P=0.045) in HPV positive samples. SLPI and IL-6 levels also lower in HPV negative- compared to HPV positive- patients but show no significantly difference at P<0.05. CXCL-10, IgA, and sCD163 showed comparable level between HPV negative and HPV positive samples (Figure 5B).

A.
B.

**Figure 5. Human papillomaviruses DNAs were detected in oral fluid of HIV infected patients.** A) q-PCR detected HPVs DNA expression in HIV infected samples. B) HPV negative samples showed significantly lower level of sCD14 (P<0.05) compared to HPV positive samples. SLPI and IL-6 showed trend of lower responses in HPV negative samples.
**Discussion**

In this study, we examined the expression of local oral immune mediators in HIV positive patients who received periodontal treatment and dental education at UNC hospital and had been followed up every 6 months for 24 months after first treatment. Majority of patients are male and the major route of HIV infection are men who had sex with men. In this group of study, 80% of patients were on HAART (Table 1). To be able to understand the necessity of dental care treatment in HIV patients, we separated patients into 3 groups based on HIV infection diagnostic time and dental care access. Peridontal parameters, CD4 counts, oral and plasma HIV viral load, oral bacterial counts, HPV viral load, and immune mediators were collected and compared between baselines (study entry, no periodontal treatment) to 12 months and 24 months after first periodontal treatment. We investigated both local oral immune mediators; SLPI and sIgA, and inflammatory activators that found in both oral and plasma; sCD14, IL-6, sCD163, CXCL-10.

Oral immune mediators are part of innate and adaptive immune responses to microbial infections/transmission into oral epithelial cells. The expression oral and systemic immune mediators may be utilized as a marker to follow HIV associated and non-HIV associated events in HIV infected patients. Oral innate immunity is composed of different peptides including Toll-like receptors, retinoic acid induced gamma interferon (RIG-I) like receptors, anti-inflammatory cytokines, defensins, mucins, histatins, lactoferrin, lysozyme, and SLPI. SLPI is a mucosal protein that has been shown to block HIV entry into epithelial cells (39). SLPI concentration was higher in HIV infected patients than in healthy control (40) but is not sufficient enough to predict immune status in HIV infected patients (41). Our data showed the reduced level of SLPI after periodontal diseases ablation. The similar shift of sCD14, sCD163, CXCL-10, and sIgA were also shown. Our results support previous findings shown by other groups. Tenorio et al. demonstrated the relationship between IL-6, sCD14, sTNF, and D-dimer to non-AIDS defining conditions in suppressive ART patients (8). CD14 is part of pattern recognition receptor function...
as a co-receptor for TLRs to detect bacterial lipopolysaccharide (42). In addition, IL-6 was also previously showed the relationship to HANA conditions. Elevated plasma levels of inflammatory markers sCD14, hsCRP, d-Dimer and IL-6 increased risk of death in HIV infected patients (43-46).

Interestingly, HIV-patients who maintained low level of viral load but have no improvement of CD4 counts even after HAART therapies had increased systemic CD4 counts after periodontal treatment (figure 4A-B). This group of HIV-patient is the most risky group for HIV-related morbidity and mortality. Following the improvement of periodontal parameters, levels of local oral immune mediators; SLPI and IgA, and oral and systemic immune mediators; IL6, sCD14, sCD163, CXCL10, were reduced in both saliva and plasma samples (figure 4C-D). HIV infected patients under HAART treatment are susceptible for microbial infection. The increasing incidence of oral warts in HIV patients is one of the pathological sequence that occur after prolong HAART treatment and become a major concern in long survival HIV infected patients (47, 48). The higher risk of microbial susceptibility in HIV infected patients are caused by both effects of HAART and the reduced in innate and adaptive immune responses. HAART is responsible for oral epithelial phenotype alteration. Study from Ghosh et al, found the role of epigenetic changes on the phenotype of oral epithelial cells originated from HIV infected patients who received HAART. They demonstrated the retarded growth of primary oral keratinocyte cells isolated from HIV infected patients on HAART compared to healthy subjects. The HIV-HAART oral keratinocyte cells had high level of MEK1/2 and p-p38 but low innate immune responses. Levels of histone deacetylase-1 and DNA methyltransferase were aberrant compared to normal subjects. Therefore, epigenetic changing of oral epithelial cells could be a critical factor for microbial infection in HIV-HAART patients (49). HIV protease inhibitors also showed the inhibitory effect on the viability of primary oral keratinocytes and immortalized oral keratinocyte cell lines by inhibit DNA synthesis (50). The proteonomic analysis of primary oral epithelial cells in HIV-HAART patients compared to healthy control also showed the down-
regulation of protein folding maintenance proteins and pro-inflammatory responses proteins, such as IL-1RA and heat shock proteins (51). Among other protein alterations, the down-regulation of protein related to immune responses is the major concern in HIV infected patients. The decreasing of immune responses allows for higher susceptibility of oral microbial infection.

Periodontitis is the chronic inflammation disease in oral cavity that strongly associated with HIV infection (12, 18). Periodontal treatment has been shown to provide benefits to HIV infected patients. It was previously shown that periodontal treatment improved CD4 counts in HIV infected patients (24). In our study, HIV patients who had periodontal treatment experienced improved periodontal status, CD4 counts, and reduced pro-inflammatory cytokines and immune mediators (figure 1-4). There was a positive correlation between oral HIV viral load to plasma HIV viral load (P<0.001). Oral sCD163 also showed positive correlation to plasma sCD163 (P=0.05) (Table 2) and the periodontal improvement did not relate to viral load (figure 3). According to our study, periodontal treatment is important for both HIV suppressive and non-suppressive patients, not only for down-regulate oral inflammation but also for enhance CD4 counts. In addition, periodontal treatment benefits HIV infected patients not only for reducing the local oral inflammation, it may also reduce the chronic systemic inflammation that causes HIV associated non-AIDS conditions. As shown in our study, the CD4 counts in HIV suppressed patients were increased after periodontal treatment. The underlining mechanism for periodontal treatment modulated CD4 improvement is still unclear. It is possibly that down-regulation of oral inflammation will reduce the risk of oral microbial translocation to gut and intestine thus reduces suppressive effects of chronic inflammation on de novo CD4 production. For future study, we will determine the mechanistic processes of oral microbial induced systematic inflammation and the mechanistic relationship between periodontitis and systemic CD4 counts.

Taken together, we purposed the model of periodontal treatment improved local and systemic immune mediators (figure 6). Improved CD4 counts and reduced local and systemic
immune/inflammatory markers highlight the importance of dental intervention to the oral and systemic health of the HIV infected patients.

Figure 6. Periodontal treatment improved local and systemic inflammation. At baseline in the presence of active periodontal disease there is significant inflammation and Immune activation locally and systemically. In this model it is hypothesized that a dental intervention will diminish both local and systemic markers of immune activation (sCD14, sCD163, IL-6, CXCL-10) and inflammation. Further that this would result in diminished HIV viral load and increase CD4 counts.

In addition, we also interested to determine the relationship between oral HIV and oral HPV and their roles on oral cytokines induction. HIV-positive individuals are the risk group for HPV infection (35, 52). The prevalence of oral, anal, and cervical HPV infection is high in
patients who maintain low CD4 levels even after HAART treatment (47, 52-54). Study by Kreimer et al. found about 6.3% of oral rinse of HIV infected patients presented high risk HPV infection (36). Not only high risk types HPVs that has been shown in HIV patients, the prevalence of low risk type HPV related oral wart is increased in HIV patients after access to HAART treatment (47, 55). Since HPV is well known as a major cause of cervical cancer and currently found to cause at least 30% of head and neck squamous cell carcinoma (HNSCC) (56), HIV infected patients are the risk group to develop HNSCC. HIV and HPV may interact with each other and result in more aggressive progression of both AIDS and cancer. It could be both the weak immune responses in HIV patients that drive the increasing prevalence of HPV related intraepithelial neoplasia (35) or the molecular interaction between HIV and HPV components. Vernon et al. found that HIV-1 tat protein bound to long control region of HPV, thus HIV may use this molecular interaction to drive the expression of HPV E6 and E7 oncogenes (57). Therefore, screening for oral HPVs is another test that should be promoted in HIV patients.

In conclusion, the findings from our study emphasize the necessity of oral screening dental care treatment, and periodontal care in HIV infected patients. Chronic local inflammation in oral cavity may bring to microbiome translocation and chronic systemic inflammation that drives HIV infection to severe AIDS conditions. Dental treatment should be recommended for HIV infected patients.
REFERENCES


