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WESTERN CAPE

THE ROLE OF HIGH-RISK HUMAN PAPILLOMAVIRUS IN PERIOcular CANCERS

by

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DECLARATION

I, **Amir H. Afrogheh**, declare that “**The Role of High-Risk Human Papillomavirus in Periocular Cancers**” is my original work and that all the sources that I have used or cited have been indicated and acknowledged by means of complete references, and that this document has not been submitted for degree purposes at any other academic institution.



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: 30 November 2018

DEDICATION

This thesis is dedicated to my grandmother, Touran Sarvarian, whose memory has accompanied me, giving me inspiration and support.



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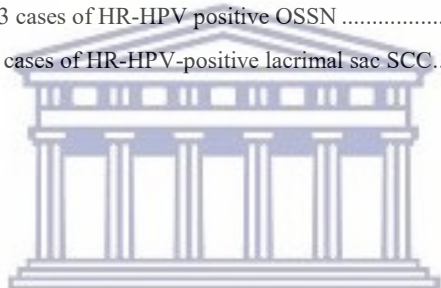
LIST OF ACRONYMS

HPV	Human Papillomavirus
HR-HPV	High-Risk Human Papillomavirus
LR-HPV	Low-Risk Human Papillomavirus
IHC	Immunohistochemistry
ISH	In-Situ Hybridization
PCR	Polymerase Chain Reaction
SC	Sebaceous Carcinoma
OSSN	Ocular Surface Squamous Neoplasia
I-SCC	Invasive Squamous Cell Carcinoma
LSSCC	Lacrimal Sac Squamous Cell Carcinoma
UVR	Ultraviolet Radiation
EV	Epidermodysplasia Verruciformis
H & E	Hematoxylin & Eosin
EVG	Elastic Von Gieson
SCCIS	Squamous Cell Carcinoma In Situ
MEC	Mucoepidermoid Carcinoma
OPSCC	Oropharyngeal Squamous Cell Carcinoma
BSC	Basal Stem Cell
FFPE	Formalin-Fixed Paraffin-Embedded
NN	Not Known
PGT	PCR-Based Genechip Technology
KS	Kaposi Sarcoma

SCJ	Squamo-Columnar Junction
TZ	Transition Zone
MGH	Massachusetts General Hospital
EUN	Emory University Hospital
NYEEI	New York Eye and Ear Infirmary
PCR-RFLP	Polymerase Chain Reaction-Restriction Fragment Length Polymorphism
ORF	Open Reading Frame
LCR	Long Control Region
IARC	International Agency Research on Cancer
pRb	Retinoblastoma
NuRD	Nucleosome Remodeling and Deacetylase complex
PBM	PDZ Binding Motif
EGFR	Epidermal Growth Factor Receptor
FRP	Florid Respiratory Papillomatosis
CDC	Center for Disease Control and Prevention
US	United States
HNSCC	Head and Neck Squamous Cell Carcinoma
AJCC	American Joint Cancer Committee
PD-1	Pre-Programmed Cell Death-1
CAP	College of American Pathologists
HNC	Head and Neck Cancer
ADCC	Adenoid Cystic Carcinoma

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ABSTRACT

Purpose: High risk human papillomavirus (HR-HPV) is well established as a causative agent of squamous cell carcinoma (SCC) of the oropharynx. HR-HPV has also been reported in periocular cancers and precancers, but controversy exists about its overall incidence and clinicopathologic profile. The purpose of this study is to evaluate the role of HR-HPV infection in periocular cancers and precancers, using multiple methods of detection.

Design: Retrospective observational case series with laboratory investigations.

Methods: Sequential surgical samples of 87 carcinomas (invasive SCC, SCC in situ and sebaceous carcinoma) from three different periocular sites (conjunctiva, lacrimal sac and the eyelid) diagnosed over a 15-year period (2000-2015) were selected for evaluation. Unstained paraffin sections of 87 cases of periocular carcinomas were analyzed with immunohistochemistry (IHC) for p16 as a screening test.

p16 positive conjunctival- and lacrimal sac SCC were further evaluated for HR-HPV using DNA in situ hybridization (DNA ISH), and a subset was also analyzed by DNA Polymerase Chain Reaction (DNA PCR). p16 positive periocular sebaceous carcinomas (SC) were analyzed with PCR, and a subset of 18 cases was further studied with a novel method of mRNA ISH, an advanced technique with an enhanced sensitivity and specificity. Relevant patient clinical information was obtained from review of the electronic medical records.

Results: Of 43 ocular surface squamous cell neoplasia (OSSN), 30% (n=13; 8 SCC in situ and 5 invasive SCC), were positive for HR-HPV. The HPV-positive OSSN occurred in 8 men and 5 women with a mean age of 60 years (range: 39 to 94 years). HPV type 16 was detected in all conjunctival cases evaluated by DNA PCR. All 5 conjunctival invasive SCC (I-SCC) showed a non-keratinizing (n=4) or partially keratinizing (n=1) histomorphology, and were managed by simple excision. In contrast, the HPV-negative

conjunctival I-SCC were predominantly keratinizing (11 keratinizing, 2 non-keratinizing). Of 9 lacrimal sac I-SCC (LSSCC), 66.7% (n=6/9) were positive for HR-HPV by p16 and DNA ISH; HPV subtypes were HPV-16 (n=5) and HPV-58 (n=1). In addition, two p16-positive cases with negative DNA ISH results were HR-HPV positive (HPV-16 and HPV-33) when evaluated by DNA PCR, suggesting that the rate of HR-HPV positivity among the LSSCC may be as high as 89% (n=8/9). The combined group of HR-HPV positive LSSCC occurred in 4 men and 4 women with a mean age of 60 years (range: 34 to 71 years). Seven of the 8 HPV-positive LSSCC (87.5%) had a non-keratinizing or partially keratinizing histomorphology while 1 case (12.5%) was predominantly keratinizing.

Strong immunohistochemical p16 positivity was present in 29 of 35 cases of periocular SC (82.9%). The selected 18 p16-positive cases tested were negative for HR-HPV using mRNA ISH. DNA PCR was unequivocal with adequate DNA isolated in 24 cases, 23 of which were negative for HR-HPV. One case was positive for HPV type 16, which was found to be a false positive as determined by mRNA ISH negativity.

Conclusion: The presence of HR-HPV in 30% of OSSN and at least 66,7% of LSSCC confirms HR-HPV as an important etiologic agent at these sites. No evidence was found for HR-HPV as an etiologic agent in the development of periocular SC using DNA PCR, and mRNA ISH to maximize sensitivity and specificity. p16 overexpression is common in periocular SC but unrelated to HR-HPV status. Immunohistochemical testing for p16, however, can be a valuable adjunct for identifying pagetoid intraepithelial spread of disease and small invasive cellular clusters. Although p16 may be used as a surrogate marker for HR-HPV status in conjunctival- and lacrimal sac carcinomas, this interpretation of p16 positivity is not applicable to periocular SC.

Keywords: Human Papillomavirus (HPV), high risk, conjunctiva, lacrimal sac, eyelid, periocular, squamous cell carcinoma, HPV-positive, DNA PCR, DNA ISH, p16, ocular surface squamous cell neoplasia (OSSN), HIV, HIV-associated OSSN, HNSCC.

CHAPTER 1

Overview of the Study

1.1 Introduction

High risk human papilloma virus (HR-HPV) infection has been increasingly recognized as a major etiologic factor for head and neck SCC, especially a subset that arises from the oropharynx.¹⁻⁵ The presence of transcriptionally active HR-HPV in oropharyngeal squamous cell carcinoma (OPSCC) is associated in general with better overall survival than HPV-negative tumors.^{2,6-8} As a result, traditional paradigms in relation to the management of OPSCC are changing.⁹⁻¹² The presence of HR-HPV has also been reported in other head and neck sites including the sinonasal cavity where up to 21% of carcinomas are HPV-related.¹³ However, most other head and neck sites investigated for HPV-related cancer including the larynx, oral cavity, and salivary glands, have shown low rates of HR-HPV, generally less than 6% .^{14,15}

An additional head and neck site where HPV-related carcinomas have been described is the conjunctiva, lacrimal sac and the eyelid, but there is disagreement regarding the incidence of HPV-related cancers at these sites.^{16,17,26-28,18-25} Detection rates for HPV in periocular cancers and precancers have ranged from 0 to 100%.^{16,17,19,20,27,29-31} The marked variation in HPV prevalence rates between different studies can be explained in part by variations in study designs and techniques used to identify HPV in biologic specimens. Many of the previous studies used only a single detection method (e.g., in situ hybridization [ISH] alone or polymerase chain reaction [PCR] alone), sample populations sometimes included a predominance of HIV-positive patients³²⁻³⁵, and sample sizes were often small or limited.^{17,19,23,25,31,36,37} Consequently, the true incidence of HR-HPV in these periocular lesions is poorly defined.

In 2011, the World Health Organization International Agency for Research on Cancer (IARC), regarded the evidence for implicating HPV as an oncogenic agent in ocular surface squamous neoplasia (OSSN) as weak, and in its report did not mention HPV as an etiologic agent in the development of OSSN.^{1,38} The key etiologic agent in the development of conjunctival cancers and precancers is thought to be exposure to ultraviolet radiation (UVR).^{39,40} Nevertheless, the development of conjunctival invasive SCC (I-SCC) is analogous to the I-SCC of the cervix, and occurs through different grades of dysplasia, suggesting a possible role for HR-HPV in the development and progression of a subset of these lesions.

p16 overexpression has consistently been reported in a high percentage of periocular SC,⁴¹⁻⁴³ regardless of HPV status (if evaluated). In other anatomic sites such as the oropharynx, p16 positivity is interpreted to equate with the presence of HR-HPV so that further viral studies are being abandoned.

The purposes of the present study were: (1) to determine the prevalence of HR-HPV in a large series of dysplastic and malignant lesions of the conjunctiva, lacrimal sac, and the eyelids using currently accepted HPV-testing methodologies (p16 immunohistochemistry as a screening test, PCR-based HPV-typing, followed by DNA in situ hybridization and mRNA in situ hybridization in a subset). (2) to define the clinical and histomorphologic features of HPV-related periocular cancers (conjunctival- and lacrimal sac squamous cell carcinomas and their precursors, and sebaceous carcinomas), which may provide new insights into the development of these neoplastic lesions. (3) to investigate whether the reported high rate of p16 expression in periocular SC correlates with HPV positivity.

1.2 Background

1.2.1 Papillomaviruses

Papillomaviruses are species-specific and have a strict tropism for mucosal and cutaneous stratified squamous epithelial surfaces of the host.⁴⁴ Papillomaviruses are an ancient group of viruses that have co-evolved extremely slowly over millions of years with their hosts, including reptiles, birds, mammals,

marsupials and fish.^{45,46} The host specificity and the benign nature of most papillomavirus infections further attest to the long virus-host relationship.⁴⁷ Papillomaviruses usually give rise to subclinical or asymptomatic infections, however, a subset of these viruses has been shown to be the causative agent of several human cancers.⁴⁸ In this chapter, the biology, natural history, evolution and cancer association of the oncogenic HPVs are discussed.

1.2.2 Human Papillomavirus Genome Organization and Structure

Papillomaviruses are non-enveloped viruses with an icosahedral shape. Their genomes consist of small double stranded circular DNA of approximately 8000 base pairs, divided into 8 or 9 Open Reading Frames (ORF).⁴⁹ The ORFs are located in three regions within the genome: the early region encodes the E1, E2, E3, E4, E5, E6 and E7 proteins; the late region encodes the L1 and L2 structural proteins; and the long control region (LCR) contains regulatory elements that control replication and transcription. All papillomaviruses encode four core proteins: E1 and E2 are replication and transcription factors^{50,51}; and L1 and L2 are capsid proteins.^{52,53} The oncogenic HPV types encode accessory proteins: E4, E5, E6 and E7.⁵⁴⁻⁵⁸ These proteins interact with a plethora of cellular proteins to promote viral replication and immune evasion.

The viral coat is arranged into 72 capsomeres with each capsomere containing 5 L1 protein molecules (72 x 5=360 L1 protein molecules), and 1 L2 molecule (1 x 72=72 L2 molecules).⁵⁹ The capsomeres are linked together via disulfide bonds.⁵⁹⁻⁶² The minor L2 capsid protein is not completely exposed on the surface of the virus.^{63,64} During infection, the L2 molecule becomes available by adhering to the host's extracellular matrix.⁶⁵ The L1 capsid protein consists of a series of hypervariable amino acid loops, that have diverged between different papillomavirus types in response to host immune selection pressure. This has implications for the current prophylactic vaccines, which offer limited cross protection.⁴⁹

1.2.3 Diversity and Taxonomy of Human Papillomaviruses

Over 200 human papillomaviruses (HPV) have been identified and completely sequenced. They are organized into five phylogenetic genera named alpha, beta, gamma, mu and u.⁶⁶ Individual HPV types have less than 90% nucleotide sequence identity in the L1 ORF compared with any other named HPV type.⁶⁷ It is interesting to note that HPV types that share a close sequence identity can show distinct human pathologies. For example, HPV-6 that shares 85% sequence identity with HPV-11 is usually detected in anogenital warts, whereas HPV-11 is commonly identified in laryngeal papillomas. Likewise, HPV-13 that shares 78% sequence identity with HPV-6 and HPV-11 is commonly found in lesions of multifocal epithelial hyperplasia (Heck's disease).^{68,69} Tropisms are thought to be primarily dictated by the regulatory elements located within the LCR region of the viral genome.⁶⁸

1.2.4 Molecular Pathology and Infectious Life Cycle of Human Papillomavirus

HPVs infect cutaneous and mucosal sites. The site of infection and the host's local microenvironment are the major determinants of a productive and successful viral life cycle.⁷⁰ The virus infects the self-renewing basal stem cells (BSC) through a micro-abrasion/fissure in the epithelium that exposes the epithelial basement membrane.⁷¹ The strategy of infecting self-renewing cells through an open wound, not only ensures long-term viral persistence in actively dividing BSC but also promotes cellular proliferation as part of the highly organized wound healing process, which could help in the establishment of the viral infection.⁴⁴ The viral capsid binds to heparin sulfate proteoglycans (and also to alpha-6 integrins and laminin-5), on either the epithelial cell surface or basement membrane through interactions with the L1 major capsid protein. After binding to heparin sulfate proteoglycans, the capsid undergoes conformational changes, followed by furin cleavage of the minor L2 capsid protein, which increases L2 N terminus exposure.⁷² Following these binding events, the L2 minor capsid protein binds to a newly identified receptor, the annexin A2 heterotetramer.⁷³

Subsequently, the HPV virion is taken into the cell by endocytosis and is trafficked through the cytoplasm. During trafficking, the virion loses its coat (uncoating) and the viral genome, in complex with L2, is enclosed in a membrane vesicle.⁷⁴ Nuclear membrane breakdown during cell division allows the L2-genome complex to access the nucleus.^{75,76} Once in the nucleus, the L2-genome complex interacts with ND10 nuclear bodies to create a local environment conducive for viral replication and transcription.^{77,78}

Early viral transcripts, encode the E1 and E2 replication proteins to sustain limited DNA amplification.⁷⁹

There are three phases of replication in the HPV life cycle: E1 and E2 are involved in the initial phase of genome amplification. E1 encodes DNA helicase required for viral replication, and E2 recruits and loads the E1-encoded DNA helicase onto the viral replication origin.^{80,81} Next is the maintenance phase of replication, when the viral genome replicates at a constant copy number in proliferating cells, often quoted as 200 copies per infected BSC.⁴⁹ During the maintenance phase of replication, E2 fastens copies of the viral genome to the host chromatin, and ensures effective distribution of the viral genomes to the daughter cells during cell division.⁸⁰ E2 is also the principal transcriptional regulator of the virus, and may activate, or more often inactivate viral transcription.⁵¹ Therefore, E2 functions in viral replication, transcription and genome partitioning. E6 and E7 are the oncoproteins of the oncogenic HPV, and are more sophisticated than the viral core proteins. E6 and E7 proteins are essential and sufficient for HPV-mediated oncogenesis. All HPVs drive cellular proliferation in the upper epithelial layers to enhance viral DNA amplification, however, E6 and E7 interact with a range of cellular proteins to promote cell cycle entry in the basal epithelial layer, leading to genetic instability of the BSC.⁸²

E7 targets retinoblastoma tumour suppressor protein (pRb), and more specifically binds and degrades p105, p107 which regulate cell cycle entry in the basal layer, as well as p130 which controls cell cycle re-entry in the upper epithelial layers.⁸³⁻⁸⁵ E7 interacts with Mi2beta, a component of the Nucleosome Remodeling and Deacetylase (NuRD) complex, with subsequent transcription of E2F genes and suppression of pRb-induced cell cycle arrest.^{86,87} E7 also activates histone demethylases, KDM6A and KDM6B, which are

involved in the induction of p16, a surrogate biomarker of oncogenic HPV infection.^{88,89} E6 conserves the telomere integrity during cell division and mediates proteasome-dependant degradation of p53 tumour suppressor protein, to encourage keratinocyte immortalization and longevity.⁹⁰⁻⁹² The PDZ Binding Motif (PBM) is situated at the extreme C terminus of the E6 protein.^{93,94} The PBM interacts with a group of PDZ binding substrates that are involved in cellular adhesion and differentiation, leading to their proteasome-mediated degradation.^{95,96} The PDZ binding activity of E6 also results in expression of cyclin B and progression of the cell cycle (G2 to M transition).^{97,98} It is also required for the regulation of the viral life cycle as well as for the episomal maintenance of HPV-31 and HPV-16 genomes in infected cells.⁹⁹⁻¹⁰¹ E6 and E7 also disrupt interferon and NFκB signalling pathways, allowing the virus to persist and evade detection.¹⁰²

E5 can facilitate immune evasion by downregulating cell surface antigen expression. E5 also stabilizes the Epidermal Growth Factor Receptor (EGFR) and thus promotes cellular proliferation by enhancing EGFR signalling pathways.⁵⁵ E5 also has a pore forming ability, inducing koilocyte formation.¹⁰³ In the superficial epithelial layers, viral DNA is amplified to a high copy number. High E4 levels are detected in the superficial keratinocytes.⁵⁴ The abundant E4 protein is arranged into amyloid fibrils, with disruption of the existing network of keratin filaments, promoting virus release and transmission.¹⁰⁴⁻¹⁰⁶ Encapsidation of the viral genome occurs through the expression of the minor coat protein, L2, and later the major coat protein, L1.^{107,108} Virus maturation occurs in the superficial dying cells, with the formation of disulfide bonds between the L1 molecules.^{109,110} Superficial keratinocytes containing a considerable number of virions are naturally sloughed from the surface of the epithelium.

1.2.5 Oncogenic Human Papillomaviruses

Over 15% of human cancers have an infectious etiology, and almost one-third of these can be attributed to infection by oncogenic HPV.¹¹¹ There has been a great deal of interest in studying HPVs, mainly because of their ability to cause cancer. Notably, cervical cancer which can result from chronic infection with a

group of oncogenic HPV types has been extensively studied.¹¹² In 2012, the IARC declared 12 HPV types as carcinogenic.¹¹³ The 12 "high-risk" human papillomaviruses (HR-HPV) belong to the alpha genus, which contains HPV types tropic to both cutaneous and mucosal epithelia. Twelve HR-HPV types have been identified as carcinogenic by the IARC (HPV types 16, 18,31,33,35,39,45,51,52,56,58 and 59).³⁸

Although "low-risk" human papillomaviruses (LR-HPV) are usually not associated with cancer, some may cause debilitating disease. For example, HPV-11 can cause laryngeal papillomas in children, a condition known as florid respiratory papillomatosis (FRP).¹¹⁴ In FRP, laryngeal papillomas can enlarge and obstruct the airway. Although surgical excision is the treatment of choice for FRP, the papillomas can recur and involve the lower respiratory tract and lung.

1.2.6 Human Papillomavirus-Positive Oropharyngeal Squamous Cell Carcinoma

1.2.6.1 Epidemiology

The discovery of HPV-DNA in Oropharyngeal Squamous Cell Carcinoma (OPSCC) can be traced back to the mid 1980's, but its role in the induction of malignancy was not conclusively established until 2000.¹¹⁵ In 2007, the IARC concluded that there was ample evidence to support a causal role for HPV-16 in oropharyngeal cancers.¹¹³ Since then the global burden of the disease has gradually increased and it has been anticipated to surpass cervical cancer in some developed countries.¹¹⁶ The Centers for Disease Control and Prevention (CDC) reported that OPSCC were one of the only five cancer types to have shown an increase in incidence from 1975 to 2009.¹¹⁷

There is significant geographic heterogeneity in the prevalence of HPV-positive OPSCC, ranging from less than 5% in India¹¹⁸, to more than 60% in North America.^{119,120} IARC estimates that there are 29000 new cases of HPV-positive OPSCC in the world each year.¹¹¹ According to CDC, there are more than 16000 cases of HPV-positive OPSCC per year in the United States (US), representing slightly over 50% of all OPSCC worldwide.¹¹⁹ Rates in Northern European countries are also high, whereas in other regions

of Europe are between 15% to 30%.¹²¹ In England, it has been estimated that the incidence of OPSCC will increase by 239% from 2011 to 2025, at which point OPSCC would comprise 35% of all Head and Neck Squamous Cell Carcinomas (HNSCC).¹¹¹ Rates are more variable in other parts of the world but are significantly lower than the rates reported in US and Northern Europe. For example, the rates are 36% and 17% for South America and Asia, respectively.¹²²⁻¹²⁴

Interestingly, over the last several decades, the reduction in tonsillectomy rates appear to have contributed to a rise in tonsillar cancer.¹²⁵ A 60% decline in tonsillar cancer risk associated with tonsillectomy procedure was noted in a Danish study, however, the risk was unchanged for base of tongue tumours.^{126,127} Thus, it is unlikely that the declines in tonsillectomy rates could have accounted for the significant rise in rates for all OPSCC.

1.2.6.2 Exposure to HR-HPV: The Risk Factor

Smoking and alcohol are well known risk factors for oral and oropharyngeal squamous cell carcinomas. A major decline in smoking rates from 1980 to 2012 has been observed in men and women in North America and Northern Europe.¹²⁸ Over the same time period, rates of oral cavity and lung squamous cell carcinomas have dropped significantly.¹²⁸

In contrast to smoking and alcohol related oral squamous cell carcinoma, which has decreasing incidence, OPSCC has shown a dramatic increase in incidence during the past several decades.^{3,129} Like cervical cancer, epidemiologic studies have demonstrated a strong association between HR-HPV exposure and OPSCC.^{130,131} HPV-16 is the most common HR-HPV in OPSCC (prevalence rate of >80%), followed by HPV-18 (prevalence rate of 3%).¹³²

Sexual behaviour is now an established risk factor for HPV-positive OPSCC, with the lifetime number of oral sex partners, as the most important risk factor for OPSCC.^{68,133} Like smoking, oral sexual practices

(including oral-oral, oral-genital, oral-anal) differ significantly across geographic regions.¹³³ However, according to the International Head and Neck Epidemiology Consortium, the number of men and women reporting a history of oral sex has dramatically increased among recent birth cohorts in comparison to distant birth cohorts (15% to 54% of men and 13% to 69% of women born before 1930 vs. after 1960, respectively).¹³³

A significant proportion of oral oncogenic HPV infections are sexually acquired.¹³⁴ Partner studies have shown type-specific oral oncogenic HPV, to be identical with the same type identified in the oral cavity or genital tract of a sexual partner.^{135,136}

Recent studies have demonstrated that the prevalence of oral oncogenic HPV infection per sexual partner is three times greater for men than for women, with higher transmission rates from females to males.¹³⁷ The prevalence of oral oncogenic HPV infection continues to rise among men with more than 5 sexual partners, but not among women.¹³⁷ This sex difference may indicate higher seroconversion rates among women following genital oncogenic HPV infections providing greater protection against subsequent oral oncogenic HPV infections.¹³⁷ It has been shown that natural seroconversion to genital HPV-16 infection decreases the risk of subsequent infection among women by 50%.¹³⁸⁻¹⁴⁰

Other risk factors for HPV-positive OPSCC include, age less than or equal to 18 years at the time of first oral sex, history of cervical HPV infection, open mouth kissing, marijuana use, vaginal and any sex partners.¹⁴¹

It is uncertain whether smoking can potentiate the risk of developing HPV-positive OPSCC. Anantharaman et al. showed that smoking was consistently associated with increased risk of OPSCC, regardless of HPV serology status.¹⁴² New data have emerged indicating that the burden of HPV-positive OPSCC is remarkably higher in ever-smokers than non-smokers in the US.¹⁴³

1.2.6.3 Clinical and Radiological Features

Over the past several decades, OPSCC incidence rates have increased more dramatically among men than women.⁵ Recent data indicate that the incidence rates for OPSCC will continue to rise for at least 30 years among men in the US, given the broad age range affected (40 to 70 years) as well as the increased incidence observed in the age group 40 to 44 years.⁵ In the US, opposite trends have been observed for women, further stressing the frequent occurrence of OPSCC in men.⁵ Of note and in contrast to conventional smoking and alcohol related HNSCC, HPV-positive OPSCC are frequently observed in younger males (<60 years old) of higher socioeconomic status, who tend to drink or smoke less and report more oral sex partners.^{2,6-8} Compared to HPV-negative HNSCCs, HPV-positive OPSCC are smaller tumours that present with early nodal dissemination.^{4,129} Secondary primary tumours or loco-regional recurrences are infrequently seen with HPV-positive OPSCC, possibly due to the lack of field cancerization effect, since transcriptionally active HPV is not detected in the peritumoral mucosa.¹⁴⁴

On radiological examination, HPV-positive OPSCC are usually small well-defined tumours with cystic nodal involvement.¹⁴⁵ HPV-positive OPSCC are generally more responsive to radiotherapy than HPV-negative OPSCC.^{9,10} The radiosensitive nature of the HPV-positive OPSCC is directly linked to their unique molecular profile. Radiated HPV-positive OPSCC cells are more likely to undergo apoptosis due to activation of functional wild type p53, that is frequently mutated in HPV-negative OPSCC.⁹ Moreover, viral E6 and E7 oncoproteins encourage rapid progression of the radiation damaged cells, through the S phase of the cell cycle, allowing them to skip DNA repair mechanisms, further increasing their radiosensitivity.^{146,147}

1.2.6.4 Improved Survival, a Unique Feature of HPV-Positive OPSCC

Overall, patients with HPV-positive OPSCC have better clinical outcomes compared to patients with conventional HPV-negative HNSCC when treated by similar modalities.^{2,6-8} HPV status has been shown

to be an independent predictor of overall survival in OPSCC patients.⁶ Gillison et al. were one of the first to report a 74% reduction in risk of death from cancer among patients with HPV-positive OPSCC compared to patients with HPV-negative OPSCC.² A meta-analysis investigating the relationship between HR-HPV infection and overall survival in HNSCC, revealed that patients with HPV-positive OPSCC had a 28% reduced risk of death in comparison to patients with HPV-negative OPSCC.⁸ It appears that the survival benefit of patients with HPV-positive OPSCC is preserved across nearly all studies, despite significant heterogeneity in patient populations, sample size, methods of HPV detection, co-morbidity and other factors in analysis, with marked reduction in tumour progression and disease-related death.^{6,7,148-150}

The survival benefit of patients with HPV-positive OPSCC, inspired O'Sullivan et al. to propose a new TNM staging system for HPV-positive OPSCC, resembling the N system for nasopharyngeal carcinoma.¹⁵¹ O'Sullivan et al. TNM staging system is currently included in the eighth edition of American Joint Cancer Committee (AJCC) cancer staging manual.¹⁵² It interesting to note that in the proposed system, HPV-positive OPSCC were defined as OPSCC positive for the surrogate biomarker p16, and other HPV-specific tests were not used to formulate O'Sullivan et al. new staging system.

It has been suggested that, parameters such as smoking and stage, may further modify the overall survival of patients with HPV-positive OPSCC.¹⁵³ For example, a high risk of disease progression is noted in patients with T4 or N3 disease.¹⁵⁴ Overall, approximately 20% of patients with HPV-positive OPSCC have a poor prognosis, and this at risk population needs to be clearly defined.¹⁵⁵

1.2.6.5 Pathogenesis of HPV-Positive OPSCC and the Oropharyngeal Transition Zone

The pathogenesis of HPV-associated OPSCC is incompletely understood and is mainly extrapolated from the cervical cancer model. The tonsillar tissues (lingual and palatine tonsils) are hot spots for HPV-induced carcinogenesis.¹²⁰ At these sites, HR-HPV, particularly HPV-16 is detected in more than 80% of SCC.¹³² The high affinity of HPV-16 for the specialized lymphoepithelium lining the tonsillar crypts, can be

explained by the activation of critical immune check point pathways in the epithelium, creating a potential "immune-privileged" site for HR-HPV infection.¹¹ For example, the inhibitory receptor programmed cell death-1 (PD-1) and its ligand PD-L1 are strongly expressed in the reticulated epithelium lining the tonsillar crypts, resulting in reduced cytotoxic T cell response to HR-HPV antigens.¹¹ Moreover, the microanatomy of the reticulated epithelium lining the tonsillar crypts (oropharyngeal transition zone) is remarkably different from the stratified squamous epithelium, lining the surface of the tonsils. The epithelium lining the tonsillar crypts rests on a dense lymphoid infiltrate that obscures the epithelium-connective tissue interface, with basal cells arranged into small cords and strands.¹⁵⁶ At electron microscopic level, the basal cells are attached to a non-contiguous, disrupted and porous basement membrane that allows free movement of lymphocytes between the epithelium and the lymphoid stroma.¹⁵⁷ Due to the inherently porous nature of the basement membrane at this site, the exposed viral entry receptors (heparin sulphate proteoglycans, alpha-6 integrins and laminin-5) that anchor the BSC to the basement membrane are readily accessible by the HR-HPV.

Infection of the BSC is followed by viral DNA integration, replication and transcription. Disruption of the key tumour suppressor proteins, p53 and pRb, by the viral oncoproteins E6 and E7 is the major molecular event in HPV-positive OPSCC, distinct from the various smoking- induced genetic alterations and mutations observed in conventional smoking and alcohol related HNSCC.^{3,158,159} In cervical cancer, HPV integration into the host genome is associated with greater expression of E6 and E7 viral oncogenes that favour carcinogenesis, however, this process is not essential.¹⁶⁰ In OPSCC, HPV DNA integration may lead to amplification of oncogenes, disruption of tumour suppressor genes and chromosomal rearrangements, modifying the outcomes of HPV-positive OPSCC.¹⁶¹ However, recent studies have shown that the majority of OPSCC have either integrated or both integrated and episomal HR-HPV DNA with no difference in patient outcomes between the two groups.^{162,163} Most HR-HPV infections are usually cleared with a year or two.¹⁶⁴ Persistent HPV infection may directly proceed to invasive cancer in 10 to 30 years.¹⁶⁵ In contrast to cervical cancer, no precancerous lesions have been established for HPV-positive OPSCC.

1.2.6.6 Tumour Infiltrating Lymphocytes and the Role of Immune Check Point Pathways in OPSCC

A possible explanation for the survival benefit of HPV-positive OPSCC patients, may be due to the presence of HPV-antigen specific T lymphocytes.¹⁶⁶ For example, HPV-16 E7 specific T lymphocytes have been demonstrated in HPV-positive OPSCC.^{167,168} Ward et al. in a study examining 270 HPV-positive OPSCC showed that HPV-positive OPSCC with high levels of tumour infiltrating lymphocytes had significantly better outcomes compared with HPV-positive OPSCC with low levels of tumour infiltrating lymphocytes (96% vs. 59% 3 year survival).¹⁶⁹

High expression of immune check point inhibitory receptors, programmed cell death-1 (PD-1) and its ligand (PD-L1) in tumour infiltrating T lymphocytes has been described in HNSCC patients.¹⁷⁰ A 70% PD-L1 expression has been reported in HPV-positive OPSCC. It has been proposed that the production of INF-gamma by tumour infiltrating lymphocytes can promote PD-L1 expression in HPV-positive OPSCC.¹⁷¹ Based on clinical trials of immunotherapeutic agents in HNSCC, investigating HPV-positive OPSCC as a subgroup, p16 positive OPSCC may demonstrate greater response rates to the anti PD-1 agent, Nivolumab, independent of levels of PD-1 expression.¹⁷²

1.2.7 HR-HPV Testing in OPSCC

Testing for HR-HPV in OPSCC has become increasingly important, having significant implications for patient prognostication and management.¹²⁰ Moreover, ascertaining that a metastatic SCC of unknown primary to a lymph node is HR-HPV positive, strongly refers to oropharynx as the site of origin.¹²⁰ For these reasons, the College of American Pathologists (CAP) strongly recommends HR-HPV testing on all patients with newly diagnosed OPSCC, including all histologic subtypes.¹²⁰ Testing may be done on the primary tumour or on a regional lymph node metastasis when the clinical findings are compatible with a primary OPSCC.¹²⁰ However, which test or combination of tests to perform is one of the more debatable issues in head and neck pathology.

An ideal test for biologically active HPV should be highly sensitive and specific, practical (with ease of application, reproducibility and interpretation), inexpensive and strongly prognostic. At present, there are a number of HPV tests and testing strategies in use, but none of them fulfil all the criteria for an ideal test. Consequently, current HPV testing practices are extensively variable. To encourage uniform testing, the recently convened College of American Pathologists (CAP) consensus panel on HPV testing in head and neck cancer, has established a set of evidence based guidelines for HR-HPV testing in HNSCC.¹²⁰ Below, the commonly used HPV testing methodologies are discussed while highlighting the advantages and disadvantages unique to each test (summarized in Table 1.1).

1.2.7.1 Polymerase Chain Reaction

Polymerase chain reaction (PCR)-based techniques use either general consensus or HPV type-specific primers to amplify and detect even a single copy of HPV DNA.¹⁷³ Hence, this HPV testing method is extremely sensitive for the presence of HPV. However, the finding of HPV DNA is not enough to classify an OPSCC as HPV-positive, since the presence of HPV DNA may indicate a transitory infection and not a true HPV-induced oncogenic event (caused by transcriptionally/biologically active HPV). Moreover, due to the highly sensitive nature of this assay false positive results are fairly common, and stringent control of the assay conditions is necessary to avoid contamination. For these reasons, the specificity of HPV DNA PCR for transcriptionally-active HPV-positive OPSCC is rather low.^{174,175}

In contrast, detection of the HPV E6/E7 mRNA transcripts may be considered a “gold standard” for classifying a tumor as HPV-positive.^{176,177} In fact, the presence of E6/E7 mRNA indicates that HPV is not only present, but is transcriptionally active. Consequently, identification of these viral transcripts by reverse transcriptase PCR appears to be an ideal HPV testing method.¹⁷³ Recent data further stress the suboptimal specificity of HPV DNA PCR for transcriptionally-active HPV-positive OPSCC. A meta-analysis of 2099 OPSCC in the US literature by Stein et al. showed a dramatic increase in the prevalence of HR-HPV DNA by PCR from 20.9 % before 1990 to 65.4% after 2000.¹⁷⁸

When considering two additional markers of HR-HPV transcriptional activity (HR-HPV DNA plus E6/E7 mRNA and/or p16), the global incidence of HPV-associated OPSCC, ranges between 18.5% and 22.4%, significantly lower than the prevalence rate of 65.4% reported in the US.¹⁷⁹ Previously, fresh frozen tumor tissue was needed for the detection of E6/E7 mRNA using reverse transcriptase PCR, an obstacle to its routine diagnostic application. Recent advances have made it possible to effectively use this technique on formalin fixed paraffin embedded tissue (FFPE).¹⁷³ This testing, however, remains technically demanding, and its use is currently limited to research laboratories.¹⁷⁵

1.2.7.2 DNA In Situ Hybridization

DNA in situ hybridization (ISH) is a technique that allows for visualization of HPV DNA in tumour cells by microscopic examination.¹⁷³ Histologically, the presence of punctate nuclear signals in tumor cells is highly specific for integration of viral DNA into the host. Therefore, a major advantage of the DNA ISH technique is its high specificity. In addition, DNA ISH can be effectively implemented on FFPE tissue specimens using widely available automated immunohistochemistry systems.¹⁷³ However, DNA ISH has somewhat limited sensitivity, especially for tumor tissues with low viral DNA copy numbers. Thus, HPV-positive OPSCC with low viral DNA copy numbers will be negative for HPV DNA using this technique. Moreover, DNA ISH results can be difficult to interpret.¹⁷³ Significant background staining or scanty/small faint hybridization signals may hamper interpretation and may lead to false positive and false negative results. Furthermore, because DNA ISH is used to detect viral DNA and not mRNA, the technique cannot discriminate between biologically active and transitory (passenger) HPV infections.

1.2.7.3 mRNA In Situ Hybridization

mRNA in situ hybridization (RNA ISH) allows for visualization of HPV E6/E7 mRNA transcripts in the histologic context. Accordingly, mRNA ISH is highly specific for the presence of transcriptionally active HPV.¹⁸⁰⁻¹⁸² Overall, the sensitivity of mRNA ISH is much better than DNA ISH.¹⁸⁰⁻¹⁸² This is because,

transcription provides a natural amplification step, that further increases the sensitivity of them RNA ISH technique. Therefore, tumors that are negative for DNA ISH due to low viral copy numbers in the nuclei, are indeed strongly mRNA ISH positive.¹⁸⁰ mRNA ISH has also been shown to correlate more strongly with patient survival than DNA ISH. While it appears that E6/E7 mRNA ISH is the ideal test for the detection of biologically active HPV in clinical samples, it is technically challenging to perform, and its use is mainly restricted to academic laboratories. It is expected that in the near future, mRNA ISH assays will be optimized to run on the widely available immunostaining platforms, reducing its technical difficulty and turnaround time, making it a feasible option for most pathology laboratories.¹⁷³

1.2.7.4 p16 Immunohistochemistry

HPV testing is necessary for an accurate diagnosis and prognosis of patients with OPSCC. Evaluation for HPV E6/E7 mRNA transcripts is considered the gold standard to confirm the presence of transcriptionally active HPV. Although HR-HPV E6/E7 mRNA ISH is the ideal test from a purely scientific perspective, it is not routinely used in most pathology laboratories, since it is expensive and technically difficult to perform. On the other hand, p16 immunohistochemistry (IHC), a surrogate marker of HR-HPV infection, is the most cost-effective and widely employed HPV testing modality, demonstrating a high sensitivity (>90%) for the presence of transcriptionally active HPV, with a statistically similar performance to E6/E7 mRNA ISH in OPSCC.^{179,181,182}

p16 is overexpressed in tumour cells, that harbor transcriptionally active HPV because viral E7 oncoprotein targets and degrades pRb tumour suppressor protein, with subsequent transcription of E2F genes and suppression of pRb-induced cell cycle arrest¹⁸³. E7 also activates histone demethylases, KDM6A and KDM6B, which are involved in the induction of p16.^{88,89} p16 IHC appears to fulfill all the criteria listed for an ideal test. Several prospective and randomized control studies, including large numbers of patients, have demonstrated that patients with p16-positive OPSCC have considerably better outcomes than p16-negative patients.^{6,7,124,149,154,182,184,185} In fact the new TNM staging system for OPSCC was developed on

the basis of survival benefit of p16-positive patients.¹⁵¹ Based on its outstanding performance on small tissue samples, practicality, and the wealth of literature implicating p16 as an independent predictor of survival in OPSCC, the CAP now recommends HR-HPV testing by surrogate marker p16 IHC for oropharyngeal non-cytology tissue specimens.¹²¹

However, using p16 IHC as a surrogate marker for HR-HPV is not without some major limitations. Although p16 IHC is highly sensitive for the presence of transcriptionally active HPV, it is only about 85-95% specific because other molecular events such as pRb mutation in HPV-negative tumors may result in p16 overexpression.^{179,181,182} Secondly, p16 IHC should only be used as a surrogate marker of HR-HPV infection in OPSCC, where it has been proven to be strongly prognostic and associated with transcriptionally-active HPV.^{121,180,186,187}

1.2.7.5 Multimodality Testing

The application of p16 IHC as the only diagnostic test for the presence of biologically active HPV in OPSCC is debatable, since a subset of HR-HPV DNA- and mRNA negative OPSCC are p16 positive, reflecting the suboptimal specificity of p16 for the presence of transcriptionally active HPV (e.g. pRb mutations can result in p16 overexpression).^{175,188,189} Accordingly, p16 IHC should be complemented with DNA PCR or ISH-based method to prevent misclassification of an HPV-negative OPSCC, which can sometimes show p16 overexpression.^{132,175,182} A recently published meta-analysis showed that the combination of p16 IHC and HPV DNA detection (preferably DNA PCR) is the most specific method to identify HPV-positive OPSCC.¹⁹⁰ Rietbergen et al. has shown that patients with p16 positive and HPV DNA- negative OPSCC have a significantly less favorable outcome compared with p16-positive, HPV-DNA-positive OPSCC patients.¹⁹¹ For this reason, besides p16 IHC testing in OPSCC, the CAP recommends that additional HPV-specific testing may be performed at the discretion of the pathologist and/or attending clinician, or in the setting of clinical trial.¹²⁰

An advantage of the combined p16-DNA ISH approach is that it combines the high sensitivity of p16 with the specificity of DNA ISH. However, DNA ISH is not optimally sensitive, and may be falsely negative for HR-HPV DNA. For this reason, a tri-modal testing approach is recommended, where p16 IHC is initially applied as a screening test and then p16-positive OPSCC are tested by HPV DNA ISH. OPSCC that are negative by DNA ISH are then tested by HPV DNA PCR.¹⁷³ Using HR HPV mRNA as a reference test, this algorithm has been shown to correlate best with the presence or absence of transcriptionally active HPV.¹⁹² The disadvantages of a multimodality testing approach include high costs, and decreased turnaround times.¹⁷³ The various HPV testing methodologies that are commonly used in routine clinical practice are listed in Table 1.1.

1.2.8 HPV-Positive Non-Oropharyngeal Head and Neck Carcinomas

Although HPV-positive HNC are observed at non-oropharyngeal sites, the incidence of HPV-positive non-oropharyngeal HNC has largely been overestimated in the literature.¹⁹³ There are a number of factors that contribute to these remarkably high incidence rates, including: (1) the prime application of sensitive, PCR-based detection assays by a number of authors, investigating the prevalence of HR-HPV in non-oropharyngeal HNC; (2) the presence of ectopic (displaced) tonsillar tissue at non-oropharyngeal head and neck sites (e.g. floor of mouth and hypopharynx), which are potential non-oropharyngeal hot spots for HPV-induced carcinogenesis; (3) large and bulky HPV-positive OPSCC, involving adjacent contiguous anatomic structures, for which an exact site of origin cannot be ascertained clinically; (4) unrestrained tumour migration from an oropharyngeal site, across the dense lymphoid tissue track (Waldheyer's ring), to a secondary site, such as the nasopharynx. A systematic review of the literature including 16 studies that examined HPV testing in non-oropharyngeal HNC showed that the prevalence of HPV-positive carcinomas is between 5.9% to 58.3%.^{187,194-202} When RNA based methods were employed or p16 IHC was combined with a DNA PCR or ISH-based method, the rate was between 2.7% to 5.9%.^{15,203}

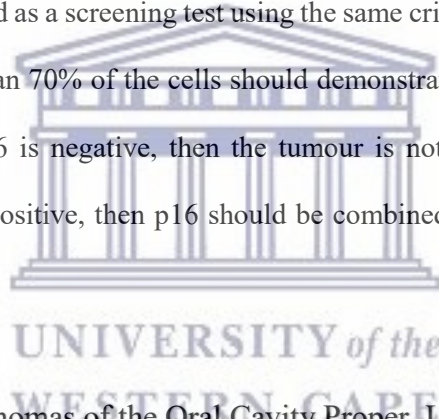
Table 1.1: Comparison of methods for detecting HPV in tissue sections

Method	Principle	Advantages	Drawbacks
Immunohistochemistry for p16	Labeled antibody targeted against p16 protein, the product of a normal tumor suppressor gene not normally expressed in non-proliferating tissues	Fast and relatively inexpensive High negative predictive value in evaluating for HPV (i.e., all HPV+ tumors are p16+)	Not specific for HPV infection (i.e., not all p16+ tumors are HPV+)
DNA in situ hybridization	Labeled DNA probe pairs with complementary HPV DNA strand in tissue sections	Able to visually localize HPV DNA to tumor cells (versus surrounding normal tissue)	Lower sensitivity (may not detect small quantities of HPV DNA) User dependent, can be difficult to interpret signal
RNA in situ hybridization	Labeled RNA probe pairs with complementary HPV RNA strand in tissue sections; signal amplified	Able to visually localize HPV DNA to tumor cells (versus surrounding normal tissue) Use of RNA probe ensures transcriptionally active HPV Bright signal Higher sensitivity due to amplification (similar to PCR)	User dependent
Polymerase chain reaction (PCR)	Amplification of HPV DNA in DNA extracted from paraffin sections followed by visualization of DNA products on polyacrylamide gel	High sensitivity (can detect minute quantities of HPV DNA)	Unable to localize HPV DNA to tumor cells; positivity may be from surrounding normal tissue or outside contaminant Time consuming

It is important to note that p16 IHC has a poor positive predictive value (PPV) in non-oro-pharyngeal HNC, especially in the oral cavity- and laryngeal SCC.¹²⁰ In addition, the biological significance for the presence of transcriptionally active HPV in HNC is largely unknown.¹²⁰ In a review of 28 studies examining the clinical outcome of patients with non-oro-pharyngeal HNC, only 5 studies showed a statistically better

survival, whereas two demonstrated that the HPV-positive group had the worse survival.¹²⁰ For these reasons, the CAP recommends that pathologists should not routinely conduct HR-HPV testing on patients with non-oropharyngeal primary tumours of the head and neck.¹²⁰ However, there are a few instances where HR-HPV testing may be indicated.¹²⁰ For example, if the anatomic site of tumour origin is not known; or when as a result of tumour migration along the Waldheyers's ring it may be clinically difficult to distinguish between an oropharyngeal and a nasopharyngeal primary (especially in large tumours); or in a patient with a history of HPV-positive OPSCC who presents with a new non-OPSCC (HPV-testing may be performed to distinguish between a new primary vs. recurrence).

In these scenarios, p16 IHC should not be used as a standalone test because of its suboptimal ppv in non-oropharyngeal HNC. It can be used as a screening test using the same criteria as in the oropharynx (for p16 to be considered positive more than 70% of the cells should demonstrate strong cytoplasmic and nuclear staining for this antibody). If p16 is negative, then the tumour is not associated with the presence of biologically active HPV. If it is positive, then p16 should be combined with a DNA PCR or ISH-based method.¹²⁰



1.2.8.1 HPV-Positive Carcinomas of the Oral Cavity Proper, Larynx and Hypopharynx

The CAP HPV testing guidelines make a clear distinction between SCC originating in the oropharynx, and those arising in the oral cavity proper, for which routine HPV-testing is not recommended.¹²⁰ The oral cavity proper includes the lips, gingiva, buccal mucosa, mobile tongue, retromolar trigone, hard palate and floor of mouth. The oropharynx includes palatine tonsils, base of tongue (posterior to circumvallate papilla), soft palate, lateral and posterior pharyngeal walls. The tonsillar structures are particularly hot spots for HR-HPV induced carcinogenic transformation, that are not present in the oral cavity proper.¹²⁰ A comprehensive review of the literature by Li et al. and Syrjanen et al. demonstrated that the rate of HPV DNA in laryngeal carcinomas was 28% and 27%.^{204,205}

A review of PCR based studies by Isayeva et al. investigating the incidence of HR-HPV at non-oropharyngeal sites revealed variable HR-HPV DNA detection rates, with rates as high as 100% and 74% for laryngeal- and oral cavity SCC respectively.²⁰⁶ In addition, a remarkably high detection rate of 70% was noted in oral samples from patients without cancer, emphasizing the need for robust testing modalities that link the presence of the virus with evidence of its biologic activity.²⁰⁶ Castellsague et al. based on testing for HPV DNA, mRNA and p16 IHC reported prevalence rates of 3%, and 1.6% for oral cavity- and laryngeal SCC respectively.¹⁴

1.2.8.2 HPV-Positive Carcinomas of the Nasopharynx

Several studies have detected transcriptionally active HR-HPV in nasopharyngeal carcinomas.²⁰⁷⁻²¹⁰ The incidence of HPV-positive nasopharyngeal carcinomas is highly variable and is largely determined by the ethnicity of the population studied and the extent of the tumour (tumour size/stage).¹⁹³ EBV-related HPV-negative non-keratinizing nasopharyngeal carcinomas are commonly seen in the Asian population, whereas HPV-positive EBV-negative non-keratinizing nasopharyngeal carcinomas are usually encountered in the North American Caucasian patients.²⁰⁷⁻²¹⁰ Moreover, HR-HPV is usually detected in advanced nasopharyngeal carcinomas. Most of these large tumours are in fact oropharyngeal tumours that have travelled across the Waldheyer's ring to secondarily involve the nasopharynx.²⁰⁹

1.2.8.3 HPV-Positive Carcinomas of the Sinonasal Tract

A comprehensive review of the literature by Li et al. and Syrjanen et al. demonstrated that the rate of HPV DNA in sinonasal carcinomas is 27%.^{204,205} Bishop et al. using p16 IHC and DNA ISH showed that 20% of the carcinomas of the sinonasal tract are associated with transcriptionally active HPV, and that this association is largely determined by the histologic type of the carcinoma studied.¹³ Like oropharynx, HR-HPV is strongly associated with a non-keratinizing morphology.¹⁹³ Moreover, certain histologic SCC types have also been reported to harbour transcriptionally active HPV, such as adenosquamous and papillary

variants.¹⁹³ Bishop et al. also described an uncommon HPV-positive sinonasal carcinoma with adenoid cystic carcinoma-like features. Unlike Adenoid Cystic Carcinomas (ADCC), these HPV-positive sinonasal carcinomas show surface intraepithelial neoplasia, and lack the MYB gene rearrangements that define head and neck ADCC.¹³ Due to the rarity of sinonasal carcinomas and the small study populations, the prognostic significance of HPV-positive sinonasal carcinomas is currently unknown. Bishop et al. has shown a strong, but statistically insignificant trend toward improved overall survival for HPV-positive sinonasal carcinomas.

1.2.9 Evidence for HPV in the Eye

1.2.9.1 HPV in Benign Conjunctival Papillomas: The Strongest Link

HPV has been strongly implicated in the pathogenesis of benign conjunctival papillomas. Conjunctival papillomas occur over a wide age range but are most common in children and young adults (20 to 39 years) with a male preponderance (60%).^{23,211} Histologically, conjunctival papillomas usually exhibit koilocytes (virally altered keratinocytes) in their superficial epithelial layers. Koilocytes are superficial or intermediate mature squamous cells with cytoplasmic clearing and nuclear pyknosis. Koilocytes are a reliable indicator of HPV infection in conjunctival papillomas.³⁷

LR-HPV types 6 and 11 are frequently detected in conjunctival papillomas, and are responsible for the development of the majority of conjunctival papillomas in children and young adults.^{29,37,211,212} Conjunctival papillomas in children are clinically distinct from adult conjunctival papillomas. They are pedunculated, often bilateral and multiple in nature.²¹³ Conjunctival papillomas in children frequently involve the conjunctival fornices or the eyelid.²¹³ An average prevalence rate of 80% for LR-HPV types 6 and 11 in conjunctival papillomas has been reported, in a review of over 200 cases.^{214,215} This implies a conclusive role for LR-HPV types 6 and 11 in the development of conjunctival papillomas. McDonnell et al. using DNA ISH technique found that 65.2% (n=15/23) of conjunctival papillomas from children and

young adults were positive for HPV-6.²¹¹ Nakamura et al. were the first in the field of ophthalmology to use a combination of HPV capsid antigen IHC, DNA-ISH and PCR to detect HPV-6 in 44% (n=4/9) of their conjunctival papillomas from children and young adults.³⁷ A higher prevalence for HPV-6 was found with both HPV IHC and DNA PCR than DNA ISH alone, suggesting that HPV IHC and DNA PCR are more sensitive assays for the detection of HPV in conjunctival papillomas.³⁷ Palazzi et al. detected HPV-11 DNA in 2 conjunctival papillomas (bilateral lesions from one patient).²¹² Tissue samples from these conjunctival papillomas were analyzed with the PCR assay, with rigorous control of the assay conditions. In a study by Eng et al. HPV-6 and 11 DNA were detected in 58% (n=14/24) of the conjunctival papillomas analyzed with nested PCR using strict measures to avoid sample contamination with amplification products.²⁹

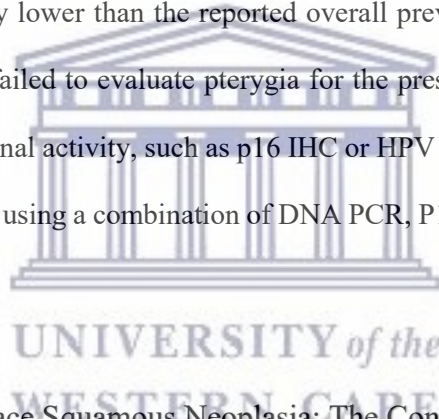
In contrast to conjunctival papillomas in children and young adults, adult conjunctival are diffuse and sessile in nature and usually involve the bulbar (ocular) conjunctiva.²¹³ Saegusa et al. using both DNA-PCR and ISH, identified HPV-16 DNA in 75% (n=12/16) of their conjunctival papillomas from adult patients, but not HPV-6 or 11.²³ The authors concluded that HPV-16 may be etiologically related to conjunctival papillomas in older adults. According to some authors, adult conjunctival papillomas in fact represent benign squamous hyperplasias or Conjunctival Intraepithelial Neoplasias (CIN).²¹³

1.2.9.2 HPV in Pterygia: The Weakest Link

Clinically, pterygia are triangular-shaped pink fleshy lesions that arise from the limbus, to cover the cornea, obstructing vision.²¹⁶ Histologically, pterygia are benign fibrovascular inflammatory lesions that exhibit degenerative (solar elastosis) and surface proliferative changes. Simple excision is the treatment of choice for pterygia, however, a few cases may recur or even display neoplastic transformation.^{217,218} The pathogenesis of pterygia is incompletely understood. There is substantial epidemiologic evidence that chronic exposure to ultraviolet radiation (UVR) is the principal risk factor for pterygia.^{26,216,219} The prevalence of pterygia in equatorial regions is as high as 23%, where high levels of UVR occur.²²⁰

An association with HPV has been proposed and demonstrated in several studies.²²¹ To elucidate the pathogenesis of pterygia, Detorakis et al. proposed a "two-hit" theory.²²² According to this theory, the development of a pterygium is mediated by two major events. The first event is mediated by the damaging effect of UVR, resulting in mutations or genetic alterations in conjunctival epithelial cells; the second event is mediated by the HPV infection of the compromised UVR-damaged conjunctival epithelial cells. This theory could be further substantiated by the idea that papillomaviruses are well known tumour promoting agents.

However, solid evidence to support a causal role for HPV in pterygia is currently lacking. An analysis of 18 studies investigating HPV in pterygia, disclosed an overall HPV prevalence rate of 19% (ranging from 0 to 100%), which is considerably lower than the reported overall prevalence rate of 34% for OSSN.²¹⁹ Furthermore, many studies have failed to evaluate pterygia for the presence of biologically active HPV, using markers of HPV transcriptional activity, such as p16 IHC or HPV E6 IHC or HPV E6/E7 mRNA. A fairly recent study from Australia, using a combination of DNA PCR, P16 IHC and HPV E6 IHC failed to detect HR-HPV in pterygia.²⁶



1.2.9.3 HPV in Ocular Surface Squamous Neoplasia: The Controversial Link

Conjunctival intraepithelial neoplasia (CIN) and invasive squamous cell carcinoma (I-SCC), collectively referred to as Ocular Surface Squamous Neoplasia (OSSN) are rare.²²³ OSSN is the third most common ocular malignancy, and represents a spectrum of atypical proliferations of the conjunctival squamous epithelium, often arising at the corneo-conjunctival junction, known as the limbus.^{219,224} Early lesions resemble pterygia or chronic conjunctivitis. In most cases, the onset is insidious with reddish grey, slightly raised lesions that may be fixed to the cornea.²²⁵ OSSN often exhibit a unilateral ocular involvement. Bilateral OSSN is usually seen in the setting of Human Immunodeficiency Virus (HIV) infection or in the context of the inherited skin disorder, Epidermodysplasia Verruciformis (EV).²²⁶⁻²²⁹

I-SCC, the most severe form of OSSN is characterized by significant ocular morbidity with the potential to recur or disseminate to regional lymph nodes. However, OSSN, in general, have a favorable outcome and death as a result of conjunctival I-SCC is a rare event. Like pterygia, OSSN arise from the limbus, and are thought to occur as a result of chronic UVR exposure.^{224,230} In fact, UVR exposure is an established risk factor for the development of OSSN. UVR is known to cause DNA damage and the formation of pyrimidine dimers.

A causal role for UVR is supported by the geographic distribution of the disease. Epidemiologic studies, using data from worldwide cancer registries, have shown a remarkably high incidence of OSSN in equatorial countries, where high levels of UVR occur.²³¹ The incidence of OSSN increases by about 50% for each 10 degrees decline in latitude.²³¹ The average incidence of OSSN is also reported to be the highest in Brisbane, Australia (1.9/100000), where it is seen primarily in the white elderly population.⁴⁰ Also, the risk of OSSN is greater in individuals with prior history of skin cancer, which is known to be caused by excessive exposure to UVR.²³⁰

Clinically, OSSN occur in sun-exposed regions of the eyes (interpalpebral region), of predominantly elderly men who engage in outdoor activities.²³² OSSN is also more common in farm workers with extended periods of exposure to UVR, dust or dirt with subsequent ocular trauma and cancer development.^{39,233,234} In rural locations, the lower incidence of OSSN among people who leave home at an earlier age, may reflect migration to metropolitan cities for work, where exposure to UVR is less.³⁹

A significant number of OSSN exhibit solar elastosis on histologic examination.^{16,235} Solar elastosis is a histological manifestation of actinic damage, where collagen, ground substance and fibroblasts in the subconjunctival squamous epithelium are replaced by abnormal elastotic material. Solar elastosis can be demonstrated in routine hematoxylin and eosin (H & E) stained sections as wavy blue-grey shredded strands of irregular sizes in the lamina propria of the conjunctival mucosa. This abnormal elastotic material stains dark brown with Elastic Van Gieson (EVG). Molecular studies have also shown some OSSN to

harbour classic UV-induced p53 mutations.^{30,34} Histologically, CIN is classified into three stages; mild (CIN I), moderate (CIN II), and severe/carcinoma in situ (CIN III) depending on the thickness of the squamous epithelium involved by abnormal squamous cells lacking maturation: CIN I-when the abnormal squamous cells involve the lower third of the conjunctival squamous epithelium; CIN II-when the abnormal cells involve two thirds of the squamous epithelium; and CIN III or squamous cell carcinoma in situ (SCCIS): when the abnormal cells involve more than two thirds or full thickness of the squamous epithelium.³⁵

I-SCC can be either keratinizing or non-keratinizing. A number of histologic variants may also be seen such as adenosquamous, lymphoepithelial, acantholytic, sarcomatoid (spindle cell) and mucoepidermoid carcinoma (MEC).²³⁶ MEC is classified as a salivary gland malignancy at extra-conjunctival head and neck sites. However, in the eye, MEC is considered to be a variant of conjunctival SCC since the conjunctival mucosa does not contain any submucosal seromucinous glands, and the tumour is thought to arise from the conjunctival surface epithelium containing mucous goblet cells.

The development of conjunctival I-SCC is analogous to the I-SCC of the cervix, which occurs through different grades of dysplasia, suggesting a possible role for HR-HPV in the development and progression of a subset of these lesions. Although DNA for HPV types 6 or 11 has been consistently identified in benign papillomas of the conjunctiva since the mid 1980's^{23,29,37,211,212}, much uncertainty exists about the role of HPV in OSSN.

The average prevalence of HPV for OSSN is greater than that reported for pterygia (33.8% vs. 18%),²¹⁹ suggesting a stronger link between OSSN and HPV. The average prevalence of HPV for OSSN, however, is significantly lower than the prevalence rate of 80% for squamous papillomas in a review of over 200 cases,^{214,215} implying a conclusive role for LR-HPV types 6 and 11 in the development of conjunctival papillomas.

There is substantial preliminary evidence that HR-HPV plays a seminal role in the development of a subset of OSSN, and a number of investigators have found a strong association between OSSN and HR-HPV.^{16,17,19,20,23–25,37,237} However, there is significant heterogeneity in the reported prevalence rates (ranging from 0 to 100%). Three studies have detected HR-HPV in 100% of the OSSN analyzed,^{17,24,25} whereas others have shown a weak or negative association.^{29–31,211,212,238,239} Of the 22 studies devoted to OSSN, only 5 have included more than 40 cases, the largest having 88. Including the current study's results, HPV 16 is the most common HR-HPV in OSSN (74.5%), followed by HPV-18 (24.2%) (Figure 1.1).

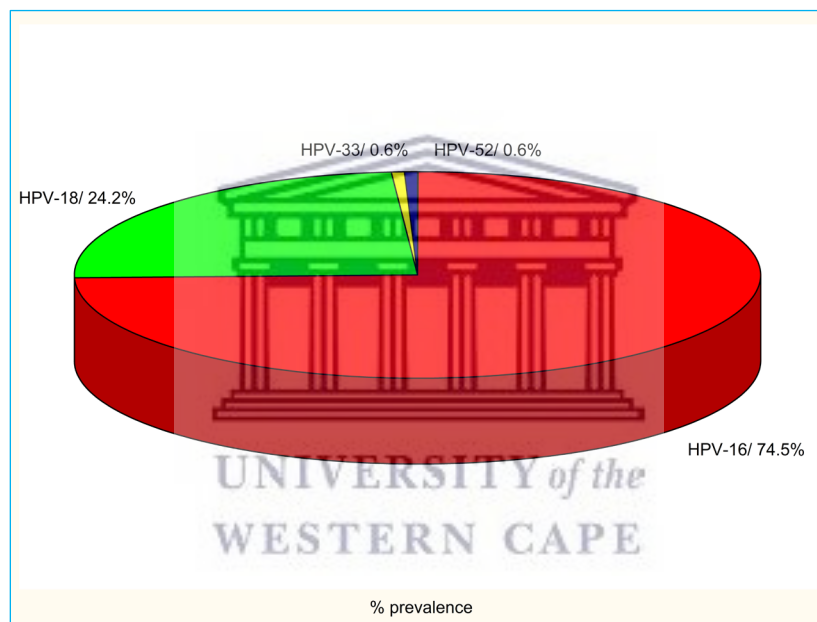


Figure 1.1: Prevalence of HR-HPV types in OSSN

The wide variation in HR-HPV prevalence rates could be explained by the different HPV testing approaches employed across individual studies (Tables 1.2-1.8). DNA PCR either alone or in combination was the most preferred and widely employed method (Tables 1.2 and 1.4). Although DNA PCR is a highly sensitive assay, this technique is not without some major limitations. A number of variables that affect sensitivity and specificity of the assay may have an impact on the reported prevalence rate. These include PCR design (broad spectrum vs. type-specific) and PCR conditions. PCR techniques using type-specific

primers may generate false negative results, since they fail to include some rare oncogenic HPV types that may be involved in the induction of OSSN. PCR is a highly sensitive method of detection, and strict control of the assay conditions are recommended to prevent cross-contamination.

The presence of confounding factors, such as HIV infection may have also contributed to the widely variable HR-HPV detection rates. For example, studies from east African countries report a high prevalence of HR-HPV infection for OSSN, given the high incidence of HIV infection in these regions.^{32,35,240} HIV is known to facilitate the activity of other oncogenic viruses, including HPV.

Finally and more importantly, only a handful of studies have confirmed their preliminary results using methods/markers of HPV transcriptional activity and to the best of our knowledge, none of the studies have used p16 as a screening test for HR-HPV as recommended by the College of American Pathologists (CAP). Thus, the role of HR-HPV in the induction of OSSN remains inconclusive. Below, an overview of the different studies is provided in a chronological order.

1.2.10 HPV-Positive OSSN in HIV-Negative Patients

1.2.10.1 DNA PCR-Based Studies of OSSN in HIV-Negative Patients

DNA PCR has been the most widely employed method for the detection of HPV in OSSN. In general, PCR is the most sensitive assay for the detection of HPV DNA. However, the application of PCR as the only HPV-specific test in OSSN has many potential pitfalls that may account for the discrepancies in the reported HPV prevalence rates. Because of its high sensitivity, false positive results are fairly common and thus stringent control of assay conditions are needed to avoid contamination. In 1989, McDonnell et al. positively identified HPV-16 DNA in a small sample of five CIN (ranging from mild to severe) and one I-SCC, from predominantly elderly male patients with a median age of 69 years.¹⁷ In addition, DNA sequences of HPV-16 were also detected in tissue swabs from both corneas of a patient with unilateral CIN.

In a subsequent study with a larger sample size (n=42), McDonnell et al. confirmed the presence of HPV-16 DNA in the majority of their OSSN (88.1%), which consisted mainly of SCCIS and I-SCC.¹⁶ Once again, HPV-16 was discovered in eyes with no grossly visible lesions, as well as in recurrent OSSN, and in successfully treated disease free eyes. The presence of HR-HPV in the apparently healthy mucosa is not unique to the eye, and has been reported in other anatomical sites, most notably, the cervix, where HR-HPV accounts for more than 95% of the cervical carcinomas.^{241,242} Approximately 15 to 40% of women with cervical HR-HPV, have no grossly visible cervical mucosal abnormalities.²⁴³

The findings of HPV-16 DNA in tissue swabs from clinically unaffected eyes, and the persistence of the virus many years following successful eradication of the lesions, suggest that HR-HPV can exist in the ocular mucosa without causing clinical disease (an insignificant passenger/transitory infection). Nevertheless, McDonnell et al. based on the presence of solar elastosis in many CIN and ISCC with detectible HR-HPV infection, assumed a secondary role for HR-HPV in the induction of OSSN, where HPV oncoproteins may act in concert with other factors (UV light or other carcinogens) to transform BSC, a multifactorial theory for ocular carcinogenesis.¹⁶ For example, an interplay between UV light and HPV appears to be an oncogenic stimulus in Epidermodysplasia Verruciformis (EV), a condition in which numerous verrucous lesions develop into papillary carcinomas in sun exposed skin.²⁴⁴

Karcioglu et al. discovered HPV-16 and 18 in 56% of their OSSN (n=25/45).²³⁷ In addition, HPV-16 and 18 were also detected in normal conjunctival mucosa and non-neoplastic conditions of the external eye, including climatic droplet keratopathy and post-traumatic corneal scars. The finding of HR-HPV in normal and non-neoplastic disorders of the ocular mucosa, prompted the authors to suggest a secondary role for HR-HPV in the development of OSSN.²³⁷ Lauer et al. investigated the presence of HR-HPV in 5 CIN with a PCR assay designed to detect the E6 region of HPV-16 and 18.¹⁹ HPV-16 DNA was found in 4 of the 5 CIN (80%). In addition, 2 of the 5 CIN showed a co-infection with HPV-18. Apart from HPV-16 and 18 infections, Tabrizi et al. demonstrated an unexpectedly high incidence (14%) (n=13/88) of HPV-6 and 11

in CIN.²⁰ Palazzi et al. demonstrated HPV-16 DNA in 9% (2/22) of their OSSN investigated with PCR.²¹² In addition, HPV-16 DNA was also present in the healthy conjunctival mucosa of a participant in the control group. Palazzi et al. pointed to the low prevalence of HR-HPV in their study sample (9%), in comparison to the high prevalence rates reported by previous studies, which could be related to the strict control of the PCR assay conditions by the authors. Palazzi et al. concluded that the low frequency of HR-HPV in OSSN and the presence of HPV-16 DNA in healthy conjunctival mucosa, do not support a causal role for HR-HPV in OSSN.²¹²

Toth et al. analyzed tissue sections obtained from 23 Formalin-Fixed Paraffin-Embedded (FFPE) conjunctival specimens for HR-HPV.²¹ 22% (n=5/23) of the cases were positive for HR-HPV (4 HPV-16 and 1 HPV-18). Eng et al. failed to detect HR-HPV in 20 OSSN with a keratinizing histomorphology, despite using a highly sensitive assay (PCR).²⁹ The negative HPV results obtained by Eng et al. could be explained by the well-differentiated keratinizing morphology of the OSSN analyzed. In general, keratinizing OPSCC are usually negative for HR-HPV, whereas non-keratinizing/undifferentiated OPSCC are commonly HR-HPV positive.^{245,246} Therefore, it is highly plausible that the same concept may be true for OSSN. Eng et al. recommended larger studies before a clear relationship between HR-HPV and histopathologic grade can be established.²⁹

Tulvatana et al. failed to demonstrate HR-HPV DNA in 28 OSSN with quantifiable DNA and amplifiable human beta globulin.²³⁵ Solar elastosis, highlighted in histologic sections by EVG, was seen in 53% of OSSN. The authors concluded that solar elastosis of the conjunctival subepithelial connective tissue is a major risk factor for OSSN in the tropical Thailand.²³⁵ Guthoff et al. used multiplex fluorescent PCR to investigate the occurrence of LR- and HR-HPV in 31 OSSN from central European patients with median age of 70 years.³⁰ The multiplex fluorescent PCR uses a set of fluorescently labelled primers to amplify the E6 and E7 regions of the HPV genome. The technique allows simultaneous detection and genotyping of 15 different HPV types (6,11, 16,18, 31,33,35,51, 52,56,58,59,68,39,45).³⁰

HPV was not detected in any of the OSSN analyzed. The absence of HR-HPV, the high prevalence of solar elastosis and the presence of p53 mutations in a subset of OSSN, indicated that chronic UVR exposure is a major risk factor for the induction of OSSN in the central European population.³⁰

Table 1.2: DNA PCR-based studies of OSSN in HIV-negative patients

Author(s)	CIN (%)	ISCC (%)	OSSN (%)	HPV-16 (%)	HPV-18 (%)	HR-HPV (%)
McDonnell et al.	6	1	7	100	0	100
McDonnell et al.	31	11	42	88.1	0	88.1
Karcioglu et al.	14	31	45	48.4	46.6	55.5
Lauer et al.	5	0	5	80	40	80
Tabrizi et al.	88	0	88	NN	NN	35.2
Palazzi et al.	12	10	22	9	0	9
Toth et al.	0	23	23	17.39	4.34	21.73
Eng et al.	6	14	20	0	0	0
Tulvatana et al.	NN	NN	28	0	0	0
Guthoff et al.	27	7	31	0	0	0
Average %						29

NN=Not Known

1.2.10.2 DNA ISH-Based Studies of OSSN in HIV-Negative Patients

DNA ISH can be useful for the detection of HPV DNA in OSSN. Radioactive ISH allows detection of viral DNA with reasonable sensitivity and specificity.^{27,211} However, this method is time consuming and requires the application of hazardous radioactive materials.²⁴⁰ On the other hand, the sensitivity of most non-radioactive ISH methods is rather limited.¹⁷³ Therefore, PCR techniques have been applied for the detection of HPV DNA in OSSN with a significantly higher sensitivity compared to other detection methodologies.^{16,17,19,20,237} Nevertheless, PCR does not permit the allocation of the detected signal to distinct morphologic alterations. In a landmark study by McDonnell et al. DNA ISH was negative for HPV-

6, 11, 16, and 18 in 28 CIN.²¹¹

Table 1.3: DNA ISH-based studies of OSSN in HIV-negative patients

Author(s)	CIN (%)	ISCC (%)	OSSN (%)	HPV-16 (%)	HPV-18 (%)	HR-HPV (%)
McDonnell et al.	0	28	28	0	0	0
Average %						0

1.2.10.3 DNA PCR and ISH-Based Studies of OSSN in HIV-Negative Patients

Tuppurainen et al. failed to detect HPV in OSSN using ISH and PCR.³¹ Dushku and co-investigators, assessed both pterygia (n=13) and limbal tumours (n=10) with DNA- PCR and ISH, but HPV was not found in any specimen.²³⁸ Saegusa et al. identified HPV-16 in 37.5% (n=3/8) and 25% (n=2/8) of the OSSN, using PCR and ISH respectively.²³ The higher prevalence of HPV-16 in OSSN investigated with PCR in comparison to ISH (37% vs. 25%), was attributed to the highly sensitive nature of the PCR assay. The authors also detected HPV-16 in conjunctival papillomas and stated that the presence of HPV-16 in both benign and malignant lesions of the conjunctiva, suggests an interdependent role for HR-HPV in the development of OSSN, acting in synergism with carcinogenic initiators such as UVR.²³

Nakamura et al. used DNA- ISH and PCR to detect HR-HPV in OSSN.³⁷ HPV-16 and 18 were identified in 42.85% (n=3/7) of the OSSN studied. A higher prevalence for HR-HPV was observed with DNA PCR than DNA ISH (42.85% vs. 25%). Nakamura et al. stated that DNA PCR is superior to DNA ISH for detection of HR-HPV in OSSN.³⁷ Manderwad et al. analyzed 57 OSSN from predominantly young (median age of 28.5 years) Indian patients for evidence of HPV DNA using type-specific PCR and a novel sensitive DNA-ISH technique, known as the ISH-CARD.²³⁹ The ISH-CARD technology is very sensitive in detecting low copies of HPV DNA.²³⁹ Standard ISH methods can detect 40 kb of DNA and 10 to 20 copies of mRNA.²⁴⁷ The CARD signal amplification technique was developed by Bobrow et al.²⁴⁸ The technique is based on the deposition of a significant number of haptenized tyramide molecules by peroxidase activity.

Several studies have successfully demonstrated the sensitivity of the ISH-CARD assay for detection of low copies of HPV DNA (as low as a single copy) in the cell or tissue preparations.²⁴⁹ Manderwad et al. found no evidence of HPV DNA in all 57 cases. These cases could be considered as truly negative, since the sensitivity of the PCR and ISH-CARD assays allows for detection of low levels of HPV DNA.^{248,249} The authors reasoned that in young Indian patients (11 patients had Xeroderma Pigmentosum, a major risk factor for OSSN), HPV is not associated with OSSN.²³⁹

Table 1.4: DNA PCR and ISH-based studies of OSSN in HIV-negative patients

Author(s)	CIN (%)	ISCC (%)	OSSN (%)	HPV-16 (%)	HPV-18 (%)	HR-HPV (%)	Other HR-HPV (%)
Tuppurainen et al.	4	0	4	0	0	0	0
Dushku et al.	4	4	8	0	0	0	0
Saegusa et al.	4	4	8	37.5	0	37.5	0
Nakamura et al.	5	2	7	14.8	28.57	42.85	0
Manderwad et al.	21	36	57	0	0	0	0
Average %				10.35	5.71	8.57	0



1.2.10.4 Reverse Transcriptase PCR-Based Studies of OSSN in HIV-Negative Patients

The demonstration of HR-HPV DNA by DNA PCR is non-specific and cannot discriminate between transitory- and transcriptionally active HPV infections. On the other hand, reverse transcriptase PCR amplification of E6/E7 mRNA is considered the gold standard for detection of clinically significant HPV infections.

Scott et al. using reverse transcriptase PCR was able to demonstrate HPV 16/18 DNA and mRNA in 10 consecutive CIN; neither HPV 16 or 18 DNA nor mRNA were detected in any of the control specimens or in any of the clinically uninvolved conjunctival specimens, as well as in clinically uninvolved conjunctival specimens from the same eyes of these patients.²⁴

Salceanu et al. demonstrated HPV-52 E6 mRNA in a moderately differentiated infiltrating keratinizing SCC from a 47 year old Romanian farmer.²⁵ The authors argued that HPV-52 is identified in approximately 4% of the cervical samples from Romanian women, and is possibly a sexually acquired infection. A cervical sample from the patient's wife showed ASC-H and was negative for HR-HPV. Evaluation of the ocular sample following excision was negative for HR-HPV.²⁵

Table 1.5: Reverse Transcriptase PCR-Based Studies of OSSN in HIV-Negative Patients

Author(s)	CIN (%)	ISCC (%)	OSSN (%)	HPV-16 (%)	HPV-18 (%)	HPV-52 (%)	HR-HPV (%)
Scott et al.	10	0	10	50	50	0	100
Salceanu et al.	0	1	1	0	0	100	100
Average %				25	25	50	100

1.2.10.5 p16 IHC as a Surrogate Marker of HR-HPV Infection in OSSN

In cervical intraepithelial neoplasia, p16 expression has been demonstrated to indicate progression into invasive carcinoma.^{250,251} p16 inhibits the progression of the cell cycle by suppressing the activity of CDK4. The latter targets pRb for phosphorylation and terminates pRb-dependant inhibition of E2F family of transcription factors, driving cell cycle progression.

In the event of HR-HPV infection, the E7 oncoprotein binds to pRb with subsequent activation of E2F and progression of the cell cycle from G1 to S phase. As a result of pRb inactivation, p16 increases via a feedback mechanism and becomes immunohistochemically detectable.^{183,184} p16 expression can be seen in normal conjunctival epithelium with stronger expression in the basal layer than the superficial epithelial layer.³⁶ The prevalence of p16 expression in healthy conjunctival mucosa ranges from 0 to 51.8%.³⁶ The percentage of p16 positive cells is significantly higher in OSSN in comparison to normal conjunctival mucosa (83.9% vs. 27.2%).³⁶

Auw-Haedrich et al. for the first time evaluated the immunohistochemical expression of p16 in 12 cases of OSSN, of which 2 CIN III were found to be positive for HPV-16 by DNA PCR.³⁶ Cells with intense nuclear and cytoplasmic staining were considered positive for p16. The percentage of p16 positive cells in each case ranged from 27 to 84%.³⁶ The highest expression (84%) was noted in one HPV-16 positive CIN III, consistent with the current view of p16 positivity in OPSCC (to be interpreted as positive, p16 immunostaining must be seen in at least 70% of tumor cells, in a nuclear and cytoplasmic distribution), for which an association with HR-HPV has been established.

As for the other HPV-positive CIN III, only 53 % of the cells were positive for p16, implying that OSSN with between 50 and 70% expression can have transcriptionally active HPV. In fact, some authors send rare cases of OPSCC with between 50 and 70% p16 expression for HPV DNA PCR or DNA ISH and if positive, consider the patients to have HPV-positive SCC.²⁵² Nevertheless, Auw-Haedrich et al. sample size is too small (n=12), on the basis of which p16 expression can be quantitatively standardized as a surrogate marker for HR-HPV in OSSN.

In the Auw-Haedrich et al. study, one p16 positive case (83% expression) was negative for HR-HPV, suggesting that other downstream alterations of the p16 pathway could also lead to p16 expression without obligating the presence of HPV.^{179,181,253} Also, no association between tumour grade and p16 expression was found, since the sample consisted predominantly of CIN with only 2 early conjunctival I-SCC.³⁶

Kuo et al. investigated the IHC expression of p16 in a small sample of 9 CIN (7 mild and 2 moderate).²⁵⁴ HPV genotyping was also performed using a DNA PCR-based genechip technology (PGT). With the PGT technology, PCR is used to amplify the corresponding part of the HPV L1 gene. The amplified product is then hybridized with an HPV genechip, providing a revert blot hybridization to detect the DNA of 39 HPV types in a single reaction.²⁵⁴ All CIN were negative for p16. However, HR-HPV were detected in 7 of the 9 CIN (77.7%) (HPV-16, 18 and 33). LR-HPV were also detected in 2 of the 9 CIN (HPV-7 and 62).

Jung et al. investigated the presence of HR-HPV in a series of conjunctival I-SCC of limbal origin from Taiwanese patients using p16 and DNA PCR.²⁵⁵ p16 immunoexpression ranged from 0 to 20%, significantly lower than the 70% threshold in OPSCC. DNA PCR results were negative for HR-HPV, suggesting that OSSN is not associated with HR-HPV infection in this geographic region.²⁵⁵

Woods et al. using both fresh frozen- and paraffin embedded tumour tissue detected HPV-16 in 6.5% of OSSN (n=3/46) analyzed with DNA PCR.²⁶ p16, HR-HPV E6 IHC, and genetic sequencing confirmed the presence of transcriptionally active HPV-16 in the 3 DNA PCR-positive early I-SCC.²⁶ The authors demonstrated a strong correlation between intense p16 immunoexpression and HPV-16 positivity. However, the very low prevalence of HPV-16 (6.5%) reported by the investigators, does not support a causal role for HR-HPV in OSSN.

Table 1.6: Studies of OSSN using p16 IHC as surrogate marker of HR-HPV infection

Author(s)	CIN (%)	ISCC (%)	OSSN (%)	HPV-16 (%)	HPV-18 (%)	HPV-33 (%)	HR-HPV (%)
Auw-Haedrich et al.	10	2	12	16.6	0	0	16.6
Kuo et al.	9	0	9	33.3	11.1	11.1	77.7
Jung et al.	0	13	13	0	0	0	0
Woods et al.	22	24	64	6.5	0	0	6.5
Average %				14.1	2.7	2.7	25.2

1.2.11 HIV-Associated HPV-Positive OSSN

The gradual deterioration of the immune system with advancing age, highlights the principal role of the immune system in the development of OSSN in the elderly population. Accordingly, long-term immunosuppression appears to be a major risk factor for the development of OSSN. OSSN has been described in the setting of systemic immunosuppression from Human Immunodeficiency Virus (HIV) infection, organ transplantation, rheumatologic disease, diabetes mellitus and ocular cicatricial

pemphigoid.^{256,257} The link between HIV infection and OSSN has been extensively studied by a number of authors, and HIV infection is now an established risk factor for the development of OSSN. The risk of OSSN in other immunosuppressed groups (e.g. tissue transplant recipients) is not entirely clear.³⁹ OSSN is relatively rare in developed countries where tissue transplantation and aggressive immunosuppressive therapies are common.³⁹

HIV-associated OSSN is relatively common in sub-Saharan Africa with a high prevalence of HIV infection. A high incidence of OSSN has been described in Rwanda, Uganda and Tanzania.²⁵⁸⁻²⁶³ In these regions, OSSN affects relatively young HIV-positive individuals. Studies from Africa suggest an 8 to 10-fold increased risk of OSSN in HIV-positive compared to HIV-negative people.^{258,260} However, the prevalence of OSSN in HIV-positive individuals is not such that, OSSN could be regarded as an Acquired Immunodeficiency Syndrome (AIDS)-defining malignancy.²⁶⁴ HIV-associated OSSN is rare in Europe and USA; the US HIV/AIDS cancer Match Registry Study (1980 to 2004) contains 15 conjunctival SCC.²⁶⁵

An association between HIV and OSSN was first reported in the late 1980's.²⁶⁶ Goedert et al. reported an increased risk of OSSN in HIV-positive patients in the US.²⁶⁷ Ateenyi-agaba et al. suggested that a high incidence of OSSN in Uganda appeared to be linked to HIV infection.²⁶⁸ A striking increase in OSSN was noted by Waddell et al. in Uganda from 1983 onwards, parallel with the rise in AIDS-defining conditions, ocular zoster and Kaposi Sarcoma (KS).²⁶⁹

Waddell et al. proposed that HIV infection is strongly associated with an increase in the incidence of OSSN in Africa and that HIV-induced immunosuppression may enhance the activity of oncogenic viruses, such as HPV.²⁶⁹ Even though with the introduction of highly active antiretroviral therapies, the incidences of opportunistic infections and AIDS-defining cancers, such as non-Hodgkin lymphoma and KS have significantly decreased, the incidences of virally-mediated non-AIDS defining malignancies, such as non-Hodgkin lymphoma, nasopharyngeal carcinoma and conjunctival SCC have been gradually increasing during the past two decades.²⁷⁰⁻²⁷³

In contrast to OSSN in HIV-negative patients, HIV-associated OSSN are clinically more aggressive with repeated loco-regional recurrences, often requiring enucleation or exenteration.^{256,274} Sun et al. described a case of HIV-associated HPV-positive OSSN in a 46-year old Caucasian woman with HCV infection.²⁷⁴ The disease recurred 4 months after initial resection of the lesion and she was subsequently managed with orbital exenteration.²⁷⁴ In general, HPV-positive OPSCC and OSSN in HIV-negative patients (whether HPV-positive or negative) have a reasonably favorable outcome, however, HIV-associated OSSN have a relatively poor prognosis, due to the profound systemic immunosuppression brought about by HIV-infection.^{256,274}

The role of HIV in the development of OSSN is unclear, and there is no supporting scientific evidence that HIV is directly responsible for inducing OSSN. Like other HIV-associated cancers, cancer risk is most probably mediated via immunosuppression.^{39,269} One plausible theory is that UVR and/or HPV and HIV are involved in a complex carcinogenic process, in which HIV infection effectively reduces the host's cellular immune response against HPV/UVR transformed limbal BSC, thus facilitating the action of HPV.^{39,269} In fact, HR-HPV has been positively identified in tissue biopsies of OSSN from HIV-positive patients, however, with highly variable results (Tables 1.7 and 1.8). HPV-16 and HPV-18 were the most common HR-HPV types detected in HIV-associated OSSN (Figure 1.2).

1.2.11.1 DNA PCR-Based Studies of OSSN in HIV-Positive Patients

Waddell et al. in a benchmark study, detected HPV-16 DNA in 35% of the conjunctival I-SSC (n=7/20) from predominantly young HIV-positive Ugandan patients. 92-95% of the I-SSC analyzed showed evidence of solar elastosis in histologic sections.²⁶⁹ Ateenyi-Agaba et al. detected EV-related HPV (12, 14, 24, 36, 37 and 38) in 21 conjunctival I-SSC (86%) from presumably HIV-positive Ugandan patients (HIV serology was unknown), suggesting a possible aetiological role for these viruses in the development of OSSN in young HIV-positive patients (mean age of 33 years).²⁷⁵

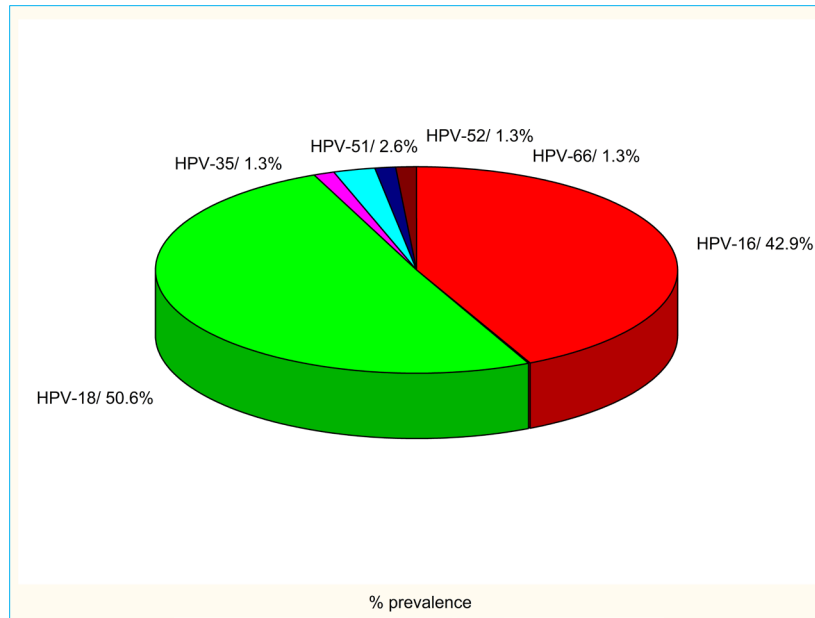


Figure 1.2: Prevalence of HR-HPV types in HIV-associated OSSN

Tornesello et al. identified HPV-6 (n=1, HIV-negative), HPV-18 (n=2, one HIV-positive), and 6 EV-related HPV [14 b (n=1, HIV-positive), 20 (n=3, 2 HIV-positive), 38 (n=1, HIV-positive), 100 (n=4, all HIV-positive), DL473 (n=1, HIV-positive), PPHL1FR (n=1, HIV-positive)] in 20% of the OSSN (n=17/86) from predominantly young HIV-positive Ugandan patients (56/86) with the mean age of 32 years.²⁷⁶ 25% (14/56) of OSSN from HIV-positive patients were HPV-positive, compared to only 10% from HIV-negative patients. A putative new HPV sequence (CJ198) was identified in 2 OSSN from HIV-positive patients.²⁷⁶

The authors concluded that EV-related HPV are frequently detected in OSSN from HIV-positive patients, concordant with several epidemiological studies, conducted by broad consensus PCR, in which EV-related HPV were commonly identified in 90% of skin lesions from immunosuppressed renal transplant recipients.²⁷⁶ de konig et al. researched the incidence of HR- and EV-related HPV in a population of mainly HIV-positive (64%) Ugandan patients and detected HR-HPV in 10 of the 81 OSSN analyzed (12.34%).³⁴ The prevalence of EV-related HPV was very low.

Yu et al. detected HPV-18 and 16 in 61% of the OSSN (n=23/38) from HIV-positive east African patients from Kenya and Uganda.³² HPV-18 was the most frequently detected HR-HPV (61%) followed by HPV-16 (16%). The authors stated that real-time quantitative PCR is a sensitive and reliable assay for the detection of specific HR-HPV in FFPE tissue.³² The largest study to determine the prevalence of HPV in OSSN from HIV-positive patients, was conducted by Ateenyi-Agaba et al.²⁷⁷ The study included 133 OSSN from predominantly young HIV-positive Ugandan patients. In this study, both frozen and formalin-fixed conjunctival biopsies were evaluated for HR- and EV-related HPV.

HPV-5 and HPV-8 were frequently detected in 50% of the cases analyzed.²⁷⁷ HPV-5 and HPV-8 belong to the beta papillomavirus genus and are usually detected in skin lesions from EV- and immunosuppressed patients.²⁷⁸ They are commonly associated with benign skin lesions but have also been isolated from malignant lesions. In fact, the early genes of HPV-8 have been demonstrated to induce both benign and malignant skin lesions in transgenic mice.²⁷⁹ However, EV-related HPV are rarely detected in OSSN, in the absence of HIV infection.²⁷⁷ Thus, the association between EV-related HPV and OSSN is most probably related to the confounding effect of HIV infection.²⁷⁷ The absence of EV-related HPV in the remaining 50% of the cases analyzed, negates a direct etiologic role for these viruses in the development of OSSN.

1.2.11.2 DNA ISH-Based Studies of OSSN in HIV-Positive Patients

Moubayed et al. used both conventional ISH and a sensitive ISH technique, ImmunoMax, to determine the presence of HPV-6,11,16 and 18 in a sample of 14 OSSN from predominantly HIV-positive Tanzanian patients.²⁴⁰ The ImmunoMax ISH for immunohistochemical analysis, includes a rigorous tissue pre-treatment phase for antigen retrieval and a unique enzyme-catalyzed amplification step.³³ The technique results in a marked increase in the sensitivity of antigen detection with no compromise in specificity.^{33,280} The ImmunoMax has also been modified for the identification of mRNA.²⁸¹

Table 1.7: DNA PCR-based studies of OSSN in HIV-positive patients

Author(s)	CIN (%)	ISCC (%)	OSSN (%)	HPV-16 (%)	HPV-18 (%)	HPV-51 (%)	HPV-66 (%)	HPV-35 (%)	HPV-52 (%)	HPV-31 (%)	HPV-33 (%)	HR-HPV (%)
Waddell et al.	0	20	20	35	0	0	0	0	0	0	0	35
Ateenyi Agaba et al.	0	21	21	0	0	0	0	0	0	0	0	0
Tornesello et al.	57	29	86	0	2.32	0	0	0	0	0	0	2.32
de koning et al.	57	24	81	9.87	1.2	2.46	1.2	1.2	1.2	0	0	16
Yu et al.	5	22	38	15.78	60.52	0	0	0	0	0	0	60.52
Ateenyi Agaba et al.	39	94	133	0	0	0	0	0	0	0	0	0
Average %												18.97



With ImmunoMax ISH, Moubayed et al. detected HPV-6,11,16 and 18 in 90% of the OSSN analyzed (n=13/14), compared to 35% (n=5/14) with traditional ISH.²⁴⁰ In all positive cases, hybridization signals were confined to the foci of CIN or ISCC. Interestingly, hybridization signals were more intense in areas of low grade CIN and the well-differentiated foci of I-SCC. This finding is in stark contrast with the high prevalence of HR-HPV in non-keratinizing/undifferentiated cervical- and oropharyngeal SCC. In addition, one of the two non-keratinizing SCC in the study was completely negative for HR-HPV DNA.²⁴⁰

Thus, it can be speculated that in HIV-positive patients both non-keratinizing and keratinizing SCC are strongly associated with HR-HPV, with a stronger association for the latter, given the high intensity of the hybridization signals seen in the well-differentiated component of the tumour. Moubayed et al. stated that the high HPV prevalence rate of 90% is comparable to the rate in cervical cancer, and is related to the geographic location and the nature of the population studied, that is immunocompromised African patients who live in subtropical Tanzania.²⁴⁰ The authors also indicated that the presence of HPV-6 and 11 in OSSN, commonly found in conjunctival papillomas, does not support a causal role for these viruses in conjunctival carcinogenesis. In fact, there is compelling evidence that a co-infection caused by HIV and HPV-16/18 is more effective in inducing OSSN than HIV and HPV-6/11.²⁴⁰

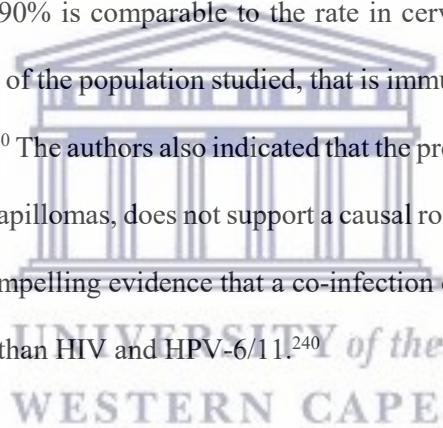


Table 1.8: DNA ISH-based studies of OSSN in HIV-positive patients

Author(s)	CIN (%)	ISCC (%)	OSSN (%)	HPV-16 (%)	HPV-18 (%)	HR-HPV (%)
Moubayed et al.	1	13	14	85.7	92.85	92.85
Average %						92.85

1.2.12 HPV and the Corneo-Conjunctival Transition Zone

In 2011, a population of residual embryonic cells were identified at the gastro-esophageal junction, linked to Barrett's metaplasia.²⁸² A subsequent study revealed a similar population of residual embryonic cells at the cervical squamocolumnar junction (SCJ), that share an identical immunophenotype (CK7 +/P63 -) with

over 90% of high grade squamous intraepithelial lesions and invasive cervical SCC, suggesting that these cells are subject to HR-HPV infection and neoplastic transformation.²⁸³ There is evidence that the SCJ cells harbour HR-HPV infection as they express HPV-16 E2 in the initial phase of the infection, and with progression to SIL show an increased expression of Ki67 proliferation index and p16.²⁸⁴ It has been shown that cryotherapeutic elimination of cervical transition zone (TZ) can reduce HPV detection rates by 50%.²⁸⁵

Similarly, OSSN usually develop at the corneo-conjunctival TZ. The corneo-conjunctival TZ, commonly referred to as the limbus, consists of a rare population of BSC, whose function is to continuously replace damaged, diseased or dead corneal cells, thereby maintaining corneal health and transparency.²⁸⁶⁻²⁸⁸ It has been postulated that the limbal BSC are more likely to undergo malignant transformation as a result of exposure to exogenous genotoxic agents, such as UVR or oncogenic HPV types.²⁸⁹ 30% OSSN are linked to HR-HPV infection. However, the number of new cervical cancer cases diagnosed annually worldwide is significantly higher than that of OSSN. The difference in cancer risk reflects morphologic differences between OSSN- and cervical TZ.²⁹⁰

The corneo-conjunctival SCJ is similar to the cervical SCJ, where corneal squamous epithelium joins the conjunctival mucosa.¹⁵⁶ In contrast to the single layered cervical SCJ, the multilayered limbal SCJ consists of basal cells and superficial columnar cells that have the ability to undergo squamous differentiation. In fact, both ocular and cervical TZ can undergo squamous change. The limbal basal cells are continuous with the basal cells of the corneal squamous epithelium. The significantly higher incidence of cervical cancer in comparison to the OSSN can possibly be explained by the multilayered structure of the ocular TZ, the infection of which requires prior injury to the epithelium for HR-HPV to gain access to the limbal BSC.

The corneo-conjunctival TZ is exposed to the external environment and is therefore prone to trauma and subsequent infection, especially when its primary protective mechanisms such as the eyelids, mucins, and tears are compromised.²¹⁹

The virus infects the self-renewing BSC through a micro-abrasion/fissure in the epithelium that exposes the epithelial basement membrane ⁷¹. The strategy of infecting self-renewing cells through an open wound, not only ensures long-term viral persistence in actively dividing BSCs but also promotes cellular proliferation as part of the highly organized wound healing process, which could help in the establishment of the viral infection⁴⁴. The viral capsid binds to heparin sulfate proteoglycans (and also to alpha6 integrins and laminin-5), on either the epithelial cell surface or basement membrane through interactions with the L1 major capsid protein. After binding to heparin sulfate proteoglycans, the capsid undergoes conformational changes, followed by furin cleavage of the minor L2 capsid protein, which increases L2 N terminus exposure ⁷². Following these binding events, the L2 minor capsid protein binds to a newly identified receptor, the annexin A2 heterotetramer ⁷³.

Subsequently, the HPV virion is taken into the cell by endocytosis and is trafficked through the cytoplasm. During trafficking, the virion loses its coat (uncoating) and the viral genome, in complex with L2, is enclosed in a membrane vesicle ⁷⁴. Nuclear membrane breakdown during cell division allows the L2-genome complex to access the nucleus ^{75,76}. Once in the nucleus, the L2-genome complex interacts with ND10 nuclear bodies to create a local environment conducive for viral replication and transcription ^{77,78}.

Early viral transcripts, encode the E1 and E2 replication proteins to sustain limited DNA amplification ⁷⁹. There are three phases of replication in the HPV life cycle: E1 and E2 are involved in the initial phase of genome amplification. E1 encodes DNA helicase required for viral replication, and E2 recruits and loads the E1-encoded DNA helicase onto the viral replication origin ^{80,81}. Next is the maintenance phase of replication, when the viral genome replicates at a constant copy number in proliferating cells, often quoted as 200 copies per infected BSC ⁴⁹. During the maintenance phase of replication, E2 fastens copies of the viral genome to the host chromatin, and ensures effective distribution of the viral genomes to the daughter cells during cell division ⁸⁰. E2 is also the principal transcriptional regulator of the virus, and may activate, or more often inactivate viral transcription ⁵¹. Therefore, E2 functions in viral replication, transcription and

genome partitioning. E6 and E7 are the oncoproteins of the oncogenic HPVs, and are more sophisticated than the viral core proteins. E6 and E7 proteins are essential and sufficient for HPV-mediated oncogenesis. All HPVs drive cellular proliferation in the upper epithelial layers to enhance viral DNA amplification, however, E6 and E7 interact with a range of cellular proteins to promote cell cycle entry in the basal epithelial layer, leading to genetic instability of the BSCs ⁸².

E7 targets Retinoblastoma tumour suppressor protein (pRb), and more specifically binds and degrades p105, p107 which regulate cell cycle entry in the basal layer, as well as p130 which controls cell cycle re-entry in the upper epithelial layers ⁸³⁻⁸⁵. E7 interacts with Mi2beta, a component of the Nucleosome Remodeling and Deacetylase (NuRD) complex, with subsequent transcription of E2F genes and suppression of pRb-induced cell cycle arrest ^{86,87}. E7 also activates histone demethylases, KDM6A and KDM6B, which are involved in the induction of p16, a surrogate biomarker of oncogenic HPV infection ^{88,89}.

E6 conserves the telomere integrity during cell division and mediates proteasome-dependant degradation of p53 tumour suppressor protein, to encourage keratinocyte immortalization and longevity ⁹⁰⁻⁹². The PDZ Binding Motif (PBM) is situated at the extreme C terminus of the E6 protein ^{93,94}. The PBM interacts with a group of PDZ binding substrates that are involved in cellular adhesion and differentiation, leading to their proteasome-mediated degradation ^{95,96}. The PDZ binding activity of E6 also results in expression of cyclin B and progression of the cell cycle (G2 to M transition) ^{97,98}. It is also required for the regulation of the viral life cycle as well as for the episomal maintenance of HPV-31 and HPV-16 genomes in infected cells ⁹⁹⁻¹⁰¹. E6 and E7 also disrupt interferon and NFkB signalling pathways, allowing the virus to persist and evade detection ¹⁰².

E5 can facilitate immune evasion by downregulating cell surface antigen expression. E5 also stabilizes the Epidermal Growth Factor Receptor (EGFR) and thus promotes cellular proliferation by enhancing EGFR signalling pathways ⁵⁵. E5 also has a pore forming ability, inducing koilocyte formation ¹⁰³.

In the superficial epithelial layers, viral DNA is amplified to a high copy number. High E4 levels are detected in the superficial keratinocytes⁵⁴. The abundant E4 protein is arranged into amyloid fibrils, with disruption of the existing network of keratin filaments, promoting virus release and transmission^{104–106}. Encapsidation of the viral genome occurs through the expression of the minor coat protein, L2, and later the major coat protein, L1^{107,108}. Virus maturation occurs in the superficial dying cells, with the formation of disulfide bonds between the L1 molecules^{109,110}. Superficial keratinocytes containing a considerable number of virions are naturally sloughed from the surface of the epithelium.

1.2.13 HR-HPV in Carcinomas of the Lacrimal Sac

In the head and neck, a causal role for HR-HPV has been established in oropharyngeal carcinomas¹¹³. HR-HPV has also been identified in conjunctival and sinonasal carcinomas.^{13,16,17,19,25} Similarly, HR-HPV has also been described in carcinomas arising from the neighbouring lacrimal sac mucosa.^{27,37,291} It has been postulated that the viral particles shed from the conjunctival neoplasms (papillomas and carcinomas) or those transmitted directly to the conjunctiva through infected objects, can reach the lacrimal sac via the tear flow.²⁷ For this reason, an association between conjunctival and lacrimal sac neoplasms may be suggested. However, conjunctival and lacrimal sac neoplasms rarely occur simultaneously.

In addition, the incidence of conjunctival papillomas and carcinomas is much higher than that of lacrimal sac papillomas and carcinomas. Unlike the lacrimal sac mucosa, the conjunctival mucosa is exposed to the external environment and is thus more prone to injury and subsequent infection. Bishop et al. using p16 IHC and DNA ISH showed that 20% of the carcinomas of the sino-nasal tract are associated with transcriptionally active HPV.¹³ Alternatively, HPV virions may travel from the nasal cavity, across the nasolacrimal duct to reach the lacrimal sac. An association between HR-HPV and carcinomas of the lacrimal sac has been proposed. However, evidence for this association is weak and is primarily based on a handful of small HPV DNA-based studies.^{27,37,291}

Madreperla et al. identified HPV in 3 of the 6 carcinomas of the lacrimal sac analyzed by DNA-PCR and ISH.²⁹¹ One case was further characterized as being HPV-18 positive, suggesting that HPV-18 may be involved in the development of the primary malignant tumours of the lacrimal sac. Sjo et al. investigated the presence of low risk- and high risk-HPV types in 4 transitional cell (non-keratinizing) - and 2 squamous cell (possibly keratinizing) carcinomas of the lacrimal sac, using a trimodal testing approach of DNA PCR, DNA ISH and RNA ISH.²⁷ HPV-16 DNA was identified in 3 of the 4 transitional cell (non-keratinizing) carcinomas by PCR.

Furthermore, two of the three HPV-16 positive carcinomas analyzed by PCR were co-infected with HPV-6 or HPV-11. Both DNA-ISH and RNA-ISH results were negative for HPV-16, clearly suggesting a transitory (passenger) HPV-16 infection or HPV-16 DNA contamination. Nevertheless, the authors suggested a strong association between HPV and carcinomas of the lacrimal sac based on the DNA PCR results, and speculated that the simultaneous occurrence of low- and high risk-HPV types in their lacrimal sac carcinomas may suggest the possibility of malignant transformation in transitional cell/inverted papillomas.²⁷ Nakamura et al. detected HPV-16 in one SCCI of the lacrimal sac using DNA ISH and PCR.³⁷

Table 1.9: Reported prevalence of HR-HPV types 16 and 18 in lacrimal sac SCCI and I-SCC

Author(s)	Year	SCCI (%)	ISCC (%)	Methods	Other HPV	HR-HPV-16 (%)	HPV-18 (%)	Overall (%)
Sjo et al.	2007	0	6	PCR, RNA ISH	None	50%	0	50%
Madreperla et al.	1993	0	6	PCR	None	0	16%	16%
Nakamura et al.	1997	1	0	ISH, PCR	None	100%	0	100%

1.2.14 Potential Targeted Therapy for HPV-Positive OSSN: HPV and EFGR

It has been shown that some viral oncoproteins can alter cellular growth factor-linked signal transduction pathways, such as those mediated by EGFR.⁵⁵ Oncoprotein E5, encoded by HPV-16 E5 gene, promotes the activation of the EGFR and its downstream signal transduction pathways via the MAP kinase activity.⁵⁵

The E7 oncoprotein encoded by HPV-16/18 E7 genes binds and inactivates the pRb tumour suppressor protein.^{87,292} The E6 oncoprotein, encoded by the HPV-16/18 E6 genes, binds to p53 tumour suppressor protein and facilitates its degradation.⁹⁰⁻⁹² The E5 oncoprotein cooperates with E7 in malignant transformation of cells and enhances the activity of E7 to induce cellular proliferation, and with E6 to immortalize cells.⁵⁵ EGFR blocking agents can improve the efficacy of chemotherapy and radiotherapy and promote tumour regression in conjunctival ISCC.

Yu et al. detected HPV-18 and 16 in 61% of the OSSN (n=23/38) from HIV-positive east African patients from Kenya and Uganda.³² HPV-18 was the most frequently detected HR-HPV (61%) followed by HPV-16 (16%). The authors stated that real-time quantitative PCR is a sensitive and reliable assay for the detection of specific HR-HPV in FFPE tissue. They also demonstrated the overexpression of EGFR and its downstream effectors, MAPK/Akt, using IHC and EGFR mRNA, in a significant proportion of HPV-positive OSSN.

This suggests a possible mechanism by which the viral oncoproteins, especially E5, may activate EGFR signal transduction, and consequently its downstream effectors, MAPK/Akt, leading to uncontrolled cell proliferation. The authors speculated that patients with HPV-positive OSSN may benefit from EGFR blocking agents.³²

Cetuximab is a chimeric monoclonal antibody which adheres to and blocks EGFR. The U.S. Food and Drug Administration has endorsed cetuximab with radiation for patients with head and neck cancer, including OPSCC. Cetuximab with radiation is an accepted standard of care, especially for patients who cannot tolerate the chemotherapeutic agent cisplatin. An interim analysis of data from a randomized clinical trial of patients with HPV-positive OPSCC has shown that treatment with radiation therapy and cetuximab is linked with worse overall and progression-free survival compared to the current standard treatment with radiation and cisplatin.²⁹³

1.2.15 HR-HPV in Periocular Sebaceous Carcinoma

Periocular sebaceous carcinoma (SC) is the third most common eyelid malignancy following basal cell carcinoma and SCC, accounting for 1% to 5.5% of all the malignancies in the periocular region.²⁹⁴⁻²⁹⁷ SC arises from the Meibomian or Zeis glands, the sebaceous glands of the eyebrow, located at the rim of the eyelids inside the tarsal plate. SC most commonly occurs in middle-aged to elderly individuals, with a female predilection.²⁹⁴ It is an aggressive tumour with frequent loco-regional recurrence and distant metastasis.²⁹⁴ A mortality rate of 30% has been reported in patients with SC.²⁹⁴

Surgical excision, radiotherapy, and chemotherapy have traditionally been employed in the management of patients with SC. The tumour generally shows minimal response to radiation treatment. The pathogenesis of periocular SC is not entirely clear. Risk factors include older age, female gender (in many studies including the current one) and prior irradiation. P53 dysregulation has been observed in a large proportion of SC.^{41,42,298,299} HER2 amplification,³⁰⁰ and epigenetic changes such as hypermethylation of the CDKN2A promoter³⁰¹ have more recently been implicated in the development of SC. The last finding has been correlated with onset at an earlier age. Recently, a few studies have shown via next-generation sequencing that SC, including periocular SC, harbor multiple concurrent mutations of RAS/RAF/MAPK and PI3K/Akt pathways.³⁰²

As a small DNA virus lacking an envelope, HPV has a distinct propensity to infect cutaneous and mucosal epithelia, generally after a micro-abrasion has removed the surface cells to allow the virus to infect deep stem cells. Meibomian glands have their glandular orifices at the mucocutaneous junction of the eyelid margin, and therefore could theoretically be directly externally infected by the virus in the same way as the conjunctiva or lacrimal sac.

A possible role for HR-HPV in the development of periocular SC was first proposed by a group of Japanese investigators in 1994.²⁸ Hayashi et al. using DNA ISH demonstrated the presence of the virus in 61.9%

(n=13/21) of periocular SC.²⁸ Of note, 57.1% (n=12/21) of the cases were associated with HR-HPV infection. Nevertheless, the authors concluded that the “positive signal in the nucleus was observed not only in the cancer cells, but also in the cells of surrounding normal sebaceous glands and epidermis...we could not determine whether HPV infections were likely to be causative or not,” clearly suggesting difficulty in interpreting test results in the setting of high background staining.²⁸

Subsequent studies have shown little or no association with HR-HPV. A report of 7 SC cases from Virginia failed to detect HPV using RNA ISH.³⁰³ In a Taiwanese study, HPV-16 was detected in only 1 of the 24 SC analyzed using nested PCR and genechip HPV typing technology.³⁰¹ The authors also demonstrated diffuse and strong immunoexpression of p16 (>50%) in a single HPV-negative SC, and stated that HR-HPV does not play a central role in the pathogenesis of SC, and that the different HPV detection methodologies used, may account for the discrepancies noted between the current study's results and those of Hayashi et al.³⁰¹

In a fairly recent South Korean study, using PANArray HPV chip test technology (PANAGENE, Daejeon, South Korea), a newly developed HPV kit with high sensitivity and specificity, HR-HPV was not identified in any of the 14 SC evaluated.³⁰⁰ However, strong and diffuse (>50%) immunoexpression of p16 was observed in 71.4% of the cases (n=10/14), consistent with the results of a similar South Korean study (79.1%), which investigated the immunoexpression of cell cycle proteins including p16 in a series of 43SC.⁴²

1.2.16 Research Context

1.2.16.1 Type of Study

The present study is a retrospective observational case series with laboratory investigations. Formalin-fixed, paraffin-embedded (FFPE) tissue blocks and haematoxylin-eosin (H & E) stained sections of 87 periocular cancers and precancers, collected from the pathology archives of three large academic hospitals:

Massachusetts General Hospital (MGH), Emory University Hospital (EUN) and New York Mass Eye and Ear Infirmary (NYEEI) were evaluated for high-risk human papillomavirus (HR-HPV).

Table 1.10: Summary of investigations of the possible role of HPV in periocular sebaceous carcinoma

Study	Number of cases	Method(s) of HPV detection	Other markers investigated	Results
Hayashi et al.	21 eyelid SC	DNA ISH	p53 (IHC)	13/21 HPV positive (61.9%) 12/21 HR-HPV positive (57.1%)
Gonzalez-Fernandez et al.	7 eyelid SC	RNAISH PCR	p53	0/7 HPV positive (0%)
Liau et al.	24 periocular SC	PCR	p16, <i>CDKN2A</i> , mismatch repair proteins	1/24 HPV positive (4.2%)
Kwon et al.	14 eyelid SC	PCR	p16, <i>KRAS</i> , <i>HER2</i>	0/12 HPV positive (0%)
Current study	35 periocular SC	PCR (24 cases) mRNA ISH (18 cases)	p16 (35 cases)	0/24 HPV positive (0%)

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1.2.16.2 Aims and Objectives

1. To determine the prevalence of HR-HPV in a large series of dysplastic and malignant lesions of the conjunctiva, lacrimal sac, and the eyelids using currently accepted HPV-testing methodologies (p16 IHC as a screening test, PCR-based HPV-typing, followed by DNA ISH and mRNA ISH in a subset).
2. To define the clinical and histomorphologic features of HPV-related periocular cancers (conjunctival and lacrimal sac SCC and their precursors, and SC of the eyelids), which may provide new insights into the development of these neoplastic lesions.
3. To investigate whether the reported high rate of p16 expression in periocular SC correlates with HPV positivity.

1.2.16.3 Hypothesis

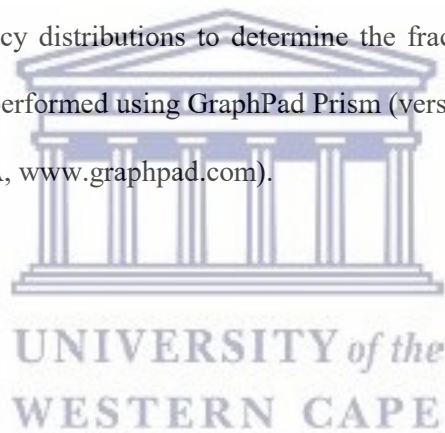
High risk human papillomavirus (HR-HPV) is present in a significant proportion of periocular cancers and precancers, suggesting the possibility of an aetiologic role for HR-HPV at these sites.

1.2.16.4 Significance of the Study

The establishment of an etiologic role for HR-HPV in periocular cancers may have major clinical and therapeutic implications in terms of management and prevention.

1.2.17 Data Analysis

Descriptive statistics and frequency distributions to determine the fraction (or percent) of HPV signal values relative to control will be performed using GraphPad Prism (version 7.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com).



CHAPTER 2

Materials and Methods

2.1 Case Selection

This study has been approved by the Institutional Review Board of the Massachusetts General Hospital (MGH), Harvard University and Partners Healthcare (IRB # 2014P00478), and Biomedical Ethics Research Committee (BMREC) of the University of the Western Cape (UWC), in compliance with the rules and regulations of the Health Insurance Portability and Accountability Act and all applicable federal and state laws, and in adherence to the tenets of the Declaration of Helsinki. Sequential surgical samples of 87 carcinomas from three different periocular sites (conjunctiva, lacrimal sac, and the eyelid) diagnosed over a 15-year period (2000-2015), were selected (I-SCC, SCCIS and SC). Basal cell carcinoma and SCC of the eyelid, and carcinomas originating from the nasal cavity, paranasal sinuses, or hard palate and involving the periocular region secondarily were excluded. The FFPE tissue blocks and H & E stained sections of these 87 cases were collected from the pathology archives of three large academic hospitals: MGH, Emory University Hospital (EUN) and New York Mass Eye and Ear Infirmary (NYEED). The H & E stained sections were reviewed to confirm the histologic diagnosis and assess the histomorphologic features of each tumour. Unstained 5- μ m FFPE sections from the surgical samples were evaluated for HR-HPV by p16 IHC, DNA ISH, DNA PCR, and mRNA ISH.

2.2 p16 Immunohistochemistry

Immunohistochemical expression of the cyclin dependent kinase inhibitor p16 was evaluated in all cases. In brief, deparaffinized FFPE sections of all cases were subjected to antigen retrieval using the Leica Bond

protocol (Leica Biosystems) with proprietary Retrieval ER2 (ethylene diamine tetraacetic acid solution, pH 9.0) for 20 minutes. A mouse monoclonal antibody against p16 (E6H4 clone, CINtec; Ventana Medical Systems, Tucson, Ariz) was utilized with a 1:4 dilution, detected by the Polymer Refine Kit (Leica Biosystems) on a Leica Bond Autostainer. For positive immunohistochemical controls, a tonsil SCC with positive p16 expression was used. The threshold for p16 positivity was met in cases where $\geq 70\%$ of tumour cells demonstrated strong diffuse nuclear and cytoplasmic staining; other staining patterns were considered negative.

2.3 HR-HPV Status by DNA ISH

All p16 positive OSSN and LSSCC (n=21) were evaluated for HR-HPV using DNA ISH. In brief, unstained slides were conditioned using Ventana cell conditioner no. 2 for 2 cycles, 12 minutes and 8 minutes, followed by ISH-protease 2 for 16 minutes. The Inform HPV III Family 16 Probe (Ventana Medical Systems), a cocktail with affinity for 12 HR-HPV genotypes (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 66) was used for hybridization, with 2 drops of probe hybridized for 2 hours. Hybridized signals were visualized with the ISH iVIEW Blue Plus Detection Kit (Ventana Medical Systems), and all reagents were obtained pre-diluted, ready for use on BenchMark Series automated slides stainers (Ventana Medical Systems). For positive ISH controls, a tonsil SCC with high HR-HPV viral DNA expression was used. The threshold for positivity was met in cases that demonstrated any number of punctate, “dot-like” hybridization signals localized to the tumor cell nuclei.

2.4 HR-HPV status by DNA PCR

All p16 positive periocular SC were evaluated with DNA PCR. PCR-based HPV typing was also performed in a subset of OSSN and LSSCC. Regions of interest were macro-dissected from 5-micron sections of FFPE tissue to enrich for tumour DNA content. For chain reaction-restriction fragment length polymorphism (PCR-RFLP), DNA was isolated using QIAamp Mini kit (Qiagen, Germany) following

manufacturer's instruction. HPV genotyping was done by a PCR-RFLP method. Briefly, a PCR reaction was first performed using HPV consensus primers designed to amplify a conserved 332-470 bp fragment of the HPV L1 gene and PCR product analyzed on a 5% polyacrylamide gel stained with ethidium bromide.

If a visible product was present, the PCR product was digested with three different restriction enzymes, Pst I, Rsa I and Hae III. The digested products were visualized on a 5% Polyacrylamide gel stained with ethidium bromide and the patterns of the digested products compared to a database of known patterns to determine the genotype. A control PCR reaction with primers to a 500-bp fragment of the human beta globin was also performed to assess the quality of the DNA.

Alternatively, HPV genotyping was performed using the HPV Linear Array assay (Roche Diagnostics). For this assay, tumour was similarly enriched using macro-dissection from 5-micron tissue sections and DNA extraction was performed with the Promega Maxwell FFPE extraction kit (Promega) according to manufacturer's protocol. HPV Linear Array was performed according to manufacturer's protocol. The sample internal control (beta-globin gene target) was used to confirm successful PCR amplification.

2.5 Automated RNA In Situ Hybridization Assays – Pooled HR-HPV and Cocktail HPV 16/18 Probes

A subset of 18 p16-positive periocular SC were evaluated using an automated RNA ISH technique. Cases were analyzed using the automated RNA ISH platform for the pooled HR-HPV assay (Cat# DVF1-17029) and cocktail of custom designed ViewRNA™ Ez probes for HPV type 16 (Cat# DVF1-17255) and HPV18 (Cat# DVF1-17256), (Affymetrix, Santa Clara, CA). The latter assay targeting specifically HPV types 16 and 18 is referred to as “cocktail HPV 16/18.” Automated ISH assays for pooled HR-HPV and cocktail HPV 16/18 mRNA were performed using ViewRNA eZ Detection Kit (Cat# QVR0001, Affymetrix) on the Bond RX immunohistochemistry and ISH Staining System with BDZ 6.0 software (Leica Biosystems, Leica Microsystems Inc., Buffalo Grove, Ill).

Freshly cut FFPE tissue sections on slides were processed automatically from deparaffinization, through ISH staining to hematoxylin counterstaining. Briefly, 5- μ m sections of FFPE tissue were baked for 1 hour at 60°C and placed on the Bond RX for processing. The Bond RX user-selectable settings were as follows: ViewRNA 1 protocol; ViewRNA Dewax1; ViewRNA HIER 10 minutes, ER1 (95); ViewRNA Enzyme 2 (20); ViewRNA Probe Hybridization for 3 hours. With these settings, the RNA unmasking conditions for the tissue consisted of a 10-minute incubation at 95°C in Bond Epitope Retrieval Solution 1 (Leica Biosystems), followed by 20-minute incubation with Proteinase K from the Bond Enzyme Pretreatment Kit at 1:1000 dilution (Leica Biosystems).

The pooled HR-HPV assay probe cocktail was diluted 1:160 in ViewRNA Probe Diluent (Affymetrix, Santa Clara, CA) and the cocktail HPV 16/18 probe mixture was diluted 1:40 each in ViewRNA probe diluent. Post run, slides were rinsed with water, air dried for 30 minutes at room temperature and mounted using Dako Ultramount (Dako, Carpinteria, CA), and visualized using a standard bright-field microscopy. As in the manual system, punctate, “dot-like” red color hybridization signals in the tumor cell nuclei and cytoplasm defined HR-HPV positivity. Additional details on the automation are provided at: http://www.panomics.com/downloads/15891_RevC%20140910_ViewRNA%20eZ%20Assay%20Manual_Compressed.pdf.

CHAPTER 3

Results

3.1 Ocular Surface Squamous Neoplasia (OSSN)

Our cohort of conjunctival ocular surface squamous neoplasms (OSSN) consisted of 25 SCCIS and 18 I-SCC comprising 33 men and 10 women with a mean age of 67 years (range: 39-94 years). Using p16 IHC as a screening test followed by DNA ISH for HR-HPV as a confirmatory test, 30% (n=13/43) of OSSN were determined to be HPV-positive (Table 11). Among these OSSN, 32% (n=8/25) of the SCCIS were positive, and 28% (n=5/18) of the I-SCC were positive (Table 3.1). Clinically, the HPV-positive OSSN occurred in 8 men and 5 women (male:female ratio 1.6:1). The male:female ratio was significantly lower than that of the HPV-negative SCC (4:1) (p=0.13062). Patients with HPV-positive OSSN had a mean age of 60 years (range: 39-94 years). By comparison, the mean age of HPV-negative OSSN was 70 years (range: 42-91 years, p=0.05937). The P-values for both the sex and age differences were >0.05 but indicate trends toward significance.

The HPV-positive SCCIS showed a characteristic histomorphology of atypical squamous cells with high N/C ratio involving the full thickness of the epithelium (Figure 3.1, A-C). The histomorphologic features of the HPV-negative SCCIS (n=17) were similar (Figure 3.1). One HPV-positive SCCIS case displayed intraepithelial keratinization (Figure 3.1, D-F) that was not detected in any of the other cases. The average size of the HPV-positive SCCIS was 0.5 cm compared to 0.9 cm for the HPV-negative SCCIS. Seven of the 8 HPV-positive SCCIS cases arose from the limbus of the corneo-conjunctival junction (Figure 3.2), and 1 case arose from the upper tarsal conjunctival epithelium. Among the HPV-positive SCCIS, two were known to be seropositive for HIV-1. The 5 HPV-positive I-SCC exhibited a non-keratinizing (n=4) or

partially keratinizing (n=1) histomorphology, while 86% of HPV-negative I-SCC were keratinizing (n=12 keratinizing, n=2; non-keratinizing).

Table 3.1: Clinicopathologic features of 13 cases of HR-HPV positive OSSN

Diagnosis	Mean Age (years)	Gender (M:F)	Partially/Non-Keratinizing	Keratinizing	P16+ & ISH+	Simple Excision	Orbital Exenteration
SCCIS (8)	57	4:4	NA	NA	8/25 (32%)	7/8 (87.5%)	1/8 (12.5%)
I-SCC(5)	63	4:1	5/5 (100%)	0	5/18 (28%)	4/5 (80%)	1/5 (20%)
OSSN(13)	60	8:5	5/5 (100%)	0	13/43 (30%)	11/13 (84.6%)	2/13 (15%)



This conventional SCCIS (A) shows diffuse positive nuclear and cytoplasmic immunoreactivity for p16 (B), and in situ hybridization for HR-HPV 16 cocktail is also positive (C). One HPV-positive SCCIS showed intraepithelial keratinization (D) and is immunohistochemically positive for p16 (E) and positive by in situ hybridization for HR-HPV 16 cocktail (F).

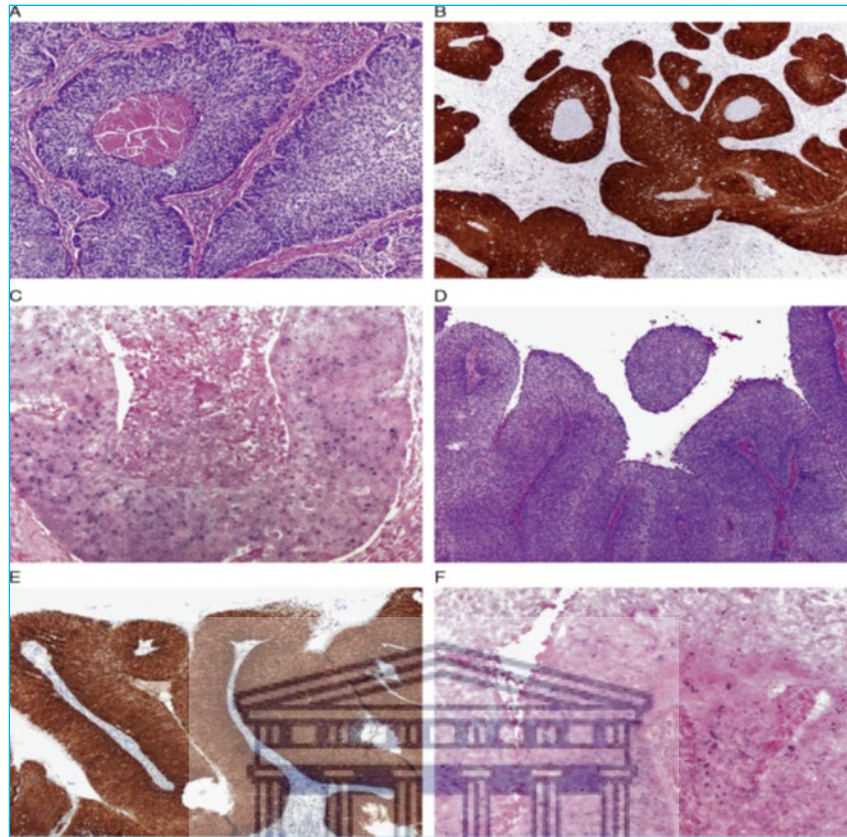
Figure 3.1: HPV-positive conjunctival SCCIS



Figure 3.2: Clinical image showing a diffuse pattern of involvement by an HR-HPV-positive SCCIS of the conjunctiva

The majority of HPV-positive I-SCC had a lobular growth pattern, a basaloid histomorphology, foci of comedo-type necrosis and a dense inflammatory background (Figure 3.3, A-C). One case of HPV-positive I-SCC with an exophytic pattern of growth was a papillary SCC (Figure 3.3, D-F). Perineural invasion was noted in one case. The average sizes of the HPV-positive I-SCC and HPV-negative I-SCC were both 1.1 cm. HPV type-16 was detected in the subset of 6 OSSN cases with informative PCR results. Three HPV-positive I-SCC cases arose from the limbus, 1 case was diffuse, and the other case occurred at the tarsus.

Similar to HPV-negative OSSN, most HPV-positive OSSN (84%; n=11/13) were primarily managed by simple excision supplemented by cryotherapy or topical chemotherapy. Local recurrence was not identified in any of the HPV-positive OSSN with available clinical information (9/13, mean follow-up period of 11 months), but distant metastasis to a periparotid lymph node was observed in one case of HPV-positive non-keratinizing I-SCC at the time of surgery. The latter case was subsequently managed by orbital exenteration, lymph node dissection, and adjuvant radiotherapy. Local recurrence was detected in 2 of the HPV-negative OSSN, both I-SCC, at 8 months and 6 years following the initial diagnosis. A distant metastasis to a periparotid lymph node was noted in one of the HPV-negative keratinizing I-SCC at 9 months following the initial diagnosis.



The carcinoma is nonkeratinizing with a lobular growth pattern and comedonecrosis (A), diffuse positivity for p16 (B), and positive by in situ hybridization for HR-HPV 16 cocktail (C). One HPV-positive case is an exophytic papillary SCC (D) that is positive for p16 (E) and in situ hybridization for HR-HPV 16 cocktail (F).

Figure 3.3: HPV-positive I-SCC of the conjunctiva

3.2 Lacrimal Sac Squamous Cell Carcinoma

Our cohort of LSSCC consisted of 9 I-SCC comprising 4 men and 5 women with a mean age of 60 years (range: 34-75 years). Using p16 IHC and DNA ISH for HR-HPV, 66.7% (N=6/9) were HPV-positive (Table 3.2). In addition, two p16-positive cases with negative DNA ISH results were HR-HPV positive (HPV-16 and HPV-33) when evaluated by PCR, suggesting that the rate of HR-HPV positivity among the LSSCC may be as high as 89% (n=8/9). The combined group of HR-HPV positive LSSCC occurred in 4 men and 4 women (male:female ratio 1:1) with a mean age of 60 years (range: 34-71 years). The lone LSSCC case which was negative for HR-HPV by p16 and ISH occurred in a female with an age of 75

years. The HPV-positive LSSCC had an average size of 1.3 cm. Seven of the 8 HPV-positive LSSCC (87.5%) had a non-keratinizing or partially keratinizing histomorphology while 1 case (12.5%) was predominantly keratinizing. The 7 non-keratinizing SCC showed a lobular growth pattern, a basaloid histomorphology, foci of comedo-type necrosis, and a dense inflammatory background (Figure 3.4, A-D). Perineural invasion was seen in the case with keratinizing histomorphology. The HPV-negative I-SCC case was non-keratinizing. HPV type-16 was detected in 6 of 8 LSSCC cases with informative PCR results. In addition, 1 case showed HPV type 33, while 1 of the 6 HPV-16 cases showed co-infection with HPV type 58. The histomorphology of these cases was similar to the others.

Six of the LSSCC cases in which clinical follow-up information was available (mean follow-up period: 5 years; range: 3.5 months to 8 years), there was a 100% overall survival rate (Table 3.2). 62.5 % of HPV-positive I-SCC with clinical follow-up was managed by radical surgical procedures including orbital exenteration and maxillectomy. Metastatic disease was not detected in any of the LSSCC, and local recurrence was noted in only one of the HPV-positive non-keratinizing SCC.

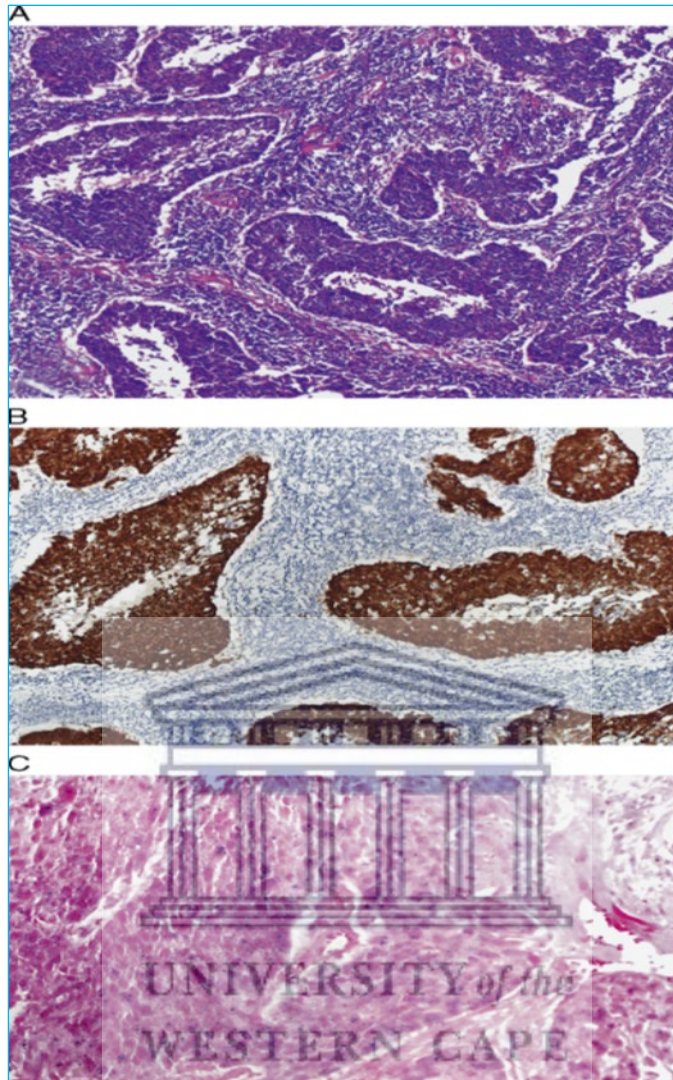
Table 3.2: Clinicopathologic features of 8 cases of HR-HPV-positive lacrimal sac SCC

Diagnosis	Mean Age (years)	Gender (M:F)	Partially/Non-keratinizing	Keratinizing	P16+ & ISH+	Excision	Exenteration or Maxillectomy
I-SCC (8)	59	4:4	7/8 (87.5%)	1/8 (12.5%)	6/9* (66.7%)	3/8 (37.5%)	5/8 (62.5%)

*Two cases were p16+/ISH negative, but positive by PCR for HR-HPV

3.3 Sebaceous Carcinoma

The cohort of periocular SC consisted of 35 cases comprising 12 men and 23 women with a mean age of 78.9 years (range, 46 to 94 y). Tumors more commonly presented in the upper eyelid (Figure 3.5, left and right). In thirteen biopsy specimens, the tumor was confined to the epithelium (Figure 3.6, top left) of the eyelid skin or conjunctiva; in the remaining 22 biopsies there was invasive disease (Figure 3.6, top right).



LSSCC. The carcinoma is non-keratinizing with lobular growth pattern in a dense inflammatory background (A); immunohistochemical staining for p16 (B) and in situ hybridization for HR-HPV 16 cocktail (C) are positive.

Figure 3.4: Immunohistochemical features of lacrimal sac squamous cell carcinoma

Ten cases contained only invasive disease in the areas sampled. Intraepithelial disease was found in the form of either carcinoma in situ or pagetoid spread of single cells. Some tumors were composed of basaloid cells containing inconspicuous vacuoles and others of sebocyte-like cells (well-differentiated cases) with well-formed vacuoles and/or a frothy appearance of the cytoplasm.

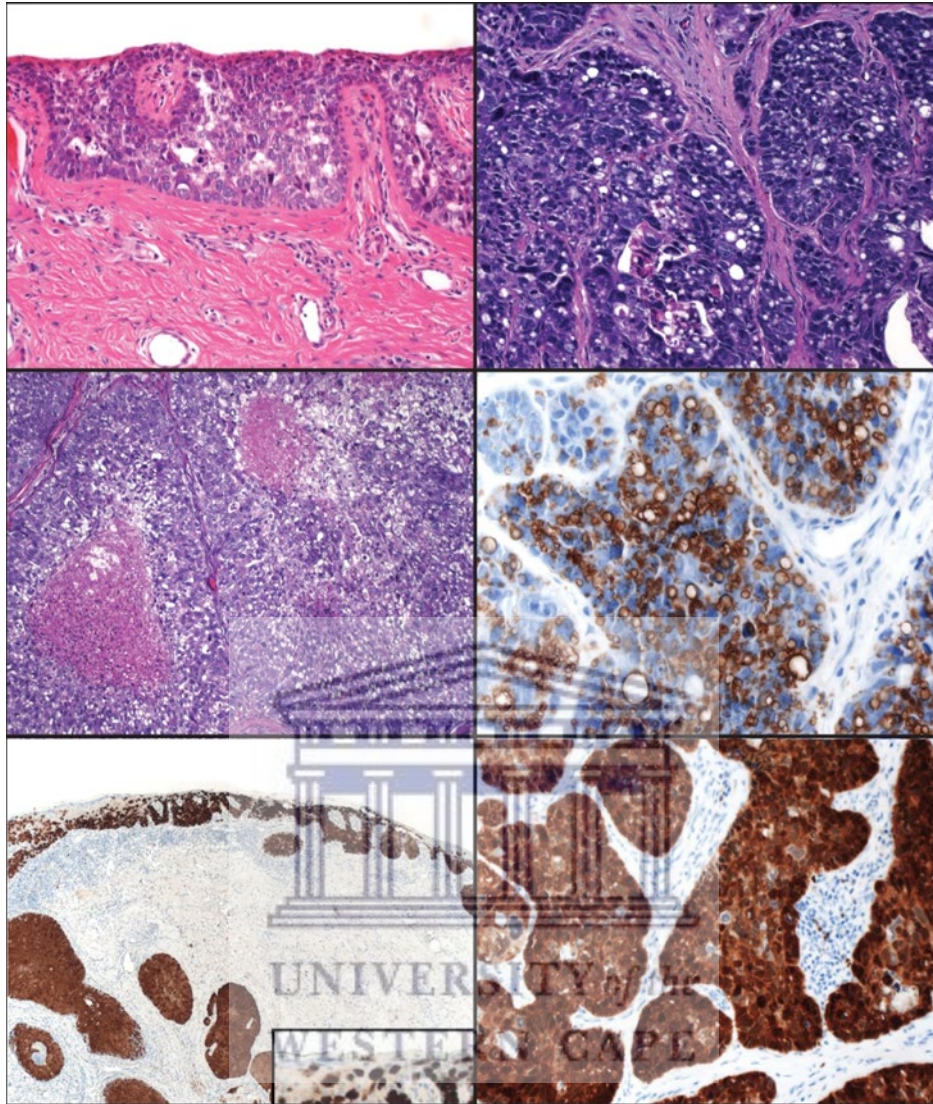


Clinical features of sebaceous carcinoma. (Left) Mild unilateral (right) eyelid margin thickening with erythema mimicking blepharitis; ptosis is also present. (Right) On eversion of the upper eyelid, a diffusely erythematous and irregular superior tarsal conjunctival surface is noted, indicative of intraepithelial (pagetoid) spread of disease.

Figure 3.5: Clinical features of sebaceous carcinoma

Comedonecrosis was a common feature of invasive tumors (Figure 3.6, middle left). Significant nuclear pleomorphism was observed including the presence of bizarre nuclei and many mitotic figures per high power field. Vesicular cytoplasmic adipophilin positivity was uniformly present (Figure 3.6, middle right). Immunohistochemistry for p16 was definitively positive in 82.9% (n = 29/35) of tumors (Figure 3.6, bottom left and right). The threshold for p16 positivity was >70% of cells with both nuclear and cytoplasmic staining. This level was used because it best correlates with HPV status in non-ophthalmic head and neck squamous tumors.⁹ Five of six of the p16-negative cases contained invasive carcinoma; one case was entirely intraepithelial in its limited sampling.

Another 3 tumors were focally or weakly positive for p16 and were categorized as p16-negative. PCR for HR-HPV was performed on all 35 tumors and was negative in 24/25 cases with adequate tumor DNA and unequivocal results; in 10 others, results were inconclusive or insufficient DNA was isolated (7 of these cases were greater than a decade old). One instance of invasive SC was positive for HPV type 16 by PCR. A subset of 18 (p16-positive) cases was studied with mRNA ISH, including the PCR-positive case, and all 18 were negative.



Sebaceous carcinoma (Top left) Vacuolated tumor cells with finely stippled nuclear chromatin display discohesiveness and replace nearly all of the tarsal conjunctival epithelium. There is an overall papillary architecture. The discohesiveness of the tumor cells contrasts with the cohesiveness of squamous neoplasms. (Top right) Invasive carcinoma cells with vacuolated cytoplasm and bizarre nuclei. (Middle left) Highly vacuolated invasive tumor cells manifest characteristic foci of comedonecrosis. (Middle right) Vesicular cytoplasmic adipophilin positivity. (Bottom left) p16 positivity of the surface epithelium and subepithelial invasive islands of tumor. The positive dermal islands represent both invasive disease and pagetoid extension within expanded adnexal structures, especially hair follicles, at the eyelid margin. The inset displays single-cell intraepithelial pagetoid tumor spread highlighted with p16. (Bottom right) p16 is diffusely positive in both the nuclei and cytoplasm of the tumor cells.

Figure 3.6: Histopathologic and immunohistochemical features of sebaceous carcinoma

CHAPTER 4

Discussion

4.1 OSSN and LSSCC

While the oropharynx remains the most common site in the head and neck where HR-HPV related SCC is known to occur, the findings from our cohort of periocular SCC and precancers suggest that the lacrimal sac and conjunctiva represent the second and third most common head and neck subsites. Although HR-HPV positive OPSCC show a characteristic strong predilection for males, the gender ratio among our cohort of patients with HR-HPV positive OSSN was only slightly higher in men (1.6:1), while in the lacrimal sac, there was an equal gender distribution (1:1). The gender ratio among all OSSN regardless of HPV status has been reported to be as great as 3:1.²³²

We did observe, however, that HR-HPV positive OSSN and LSSCC occurred at a younger mean age (60 years old) compared with HR-HPV negative OSSN (mean age of 70 years), analogous to the age difference seen in HPV-positive vs. HPV-negative OPSCC.^{2,6-8} A strong association between the presence of HR-HPV and a non-keratinizing histomorphology is well known in OPSCC.^{245,246} Similarly, in our cohort of periocular tumors, a majority of HPV-positive LSSCC and all of the conjunctival I-SCC were non-keratinizing or partially-keratinizing although over 90% of conjunctival lesions in general are known to be non-leukoplakic.²³² OSSN, comprised of dysplasias and I-SCC of the conjunctiva and cornea, is the third most common ocular malignancy, with a worldwide incidence of 0.02 to 3.5 per 100,000.²¹⁹ Most OSSN arise from the limbus (corneo-conjunctival junction).^{224,304} The etiology of OSSN is not fully understood, but epidemiological and histopathological studies indicate that UVR is among the most important risk factors for the development of OSSN.^{216,224,230} A high prevalence of OSSN has consistently been reported

in equatorial African countries, subjected to high levels of UVR.²³¹ HIV has been identified as another etiologic factor. HIV-associated OSSN is relatively common in sub-Saharan Africa with a high prevalence of HIV infection.^{35,258-263} It is rare in Europe and USA. The US HIV/AIDS cancer Match Registry Study (1980 to 2004) contains 15 conjunctival SCC.²⁶⁵ In contrast to OSSN in HIV-negative patients, HIV-associated OSSN are clinically more aggressive with repeated loco-regional recurrences, often requiring enucleation or exentration.^{256,274} It has been proposed that HIV enhances the activity of oncogenic viruses, including HPV. A number of investigators have found a high prevalence of HR-HPV in OSSN from HIV-positive patients.^{32,33,35} A review of the literature shows that HPV-18 and 16 are the most common HR-HPV in HIV-associated OSSN (Figure 1.2). Two of the patients with HR-HPV positive SCCIS in our cohort of OSSN were found to be co-infected by HIV.

A range of studies has previously reported the presence of HR-HPV in OSSN and LSSCC; however, the incidence of HPV is controversial and detection rates have varied from 0-100% (Tables 1.2-1.6 and 1.9), due in part to variations in HPV detection methods used, and sample sizes. A search of the literature identified 22 studies devoted to OSSN and 3 studies to LSSCC, that investigated the prevalence of HR-HPV in each site (Tables 1.2-1.6 and 1.9). HPV type-16 was the most prevalent genotype identified as was also found in our cohort of periocular cases (Figure 1.1). Almost one third of prior studies (n=8) failed to detect HR-HPV in OSSN,^{29-31,211,235,238,239,255} whereas 3 authors noted HPV in 100% of their cases.^{17,24,25} This marked variation in HR-HPV prevalence rates between different studies appears to be largely related to differences in detection methods used. DNA PCR, either alone or in combination (Tables 1.2 and 1.4), was the most commonly used technique.

In general, DNA PCR-based methods yielded a significantly higher detection of HR-HPV (up to 100%), in comparison to other testing modalities (Table 1.2).^{16,17,19,20} However, PCR detection alone does not distinguish HPV infections that are truly causative (i.e. transcriptionally-active) from those that are not (i.e., so-called "passenger" HPV).^{174,175} For example, in oral cancer, HR-HPV can be detected in a small

but reproducible subset of patients using PCR detection methods, yet these tumors fails to exhibit the improved outcomes that characterize HPV-related oropharyngeal cancer. On the other hand, p16 IHC, a surrogate marker of HR-HPV infection, is the most cost-effective and widely employed HPV testing modality, demonstrating a high sensitivity (>90%) for the presence of transcriptionally active HPV, with a statistically similar performance to E6/E7 mRNA ISH in OPSCC.^{179,181,182}

Given the high sensitivity of p16 as a screening test for HR-HPV, and the high specificity of DNA ISH for HR-HPV, we chose to use this combination to establish the prevalence of HR-HPV in our cohort of patients. Our study is among the few to use such a multimodal testing approach, and the first, to the best of our knowledge, to examine the incidence of HR-HPV in OSSN and LSSCC using an accepted standard combination of p16 IHC and DNA ISH.

Using this approach, we were able to establish the presence of HPV 16 in 30% and 66.7% of the OSSN and LSSCC, respectively. Our results are concordant with those of previous studies by Saeugusa and Nakamura et al.^{23,37} that have reported an incidence of 37% and 42% for OSSN using a combination of PCR and ISH techniques (Table 1.4).

In OPSCC, HPV-16 integration status has been linked to increased survival and improved outcome.^{2,6-8} Current clinical data suggest that patients with HPV-positive oropharyngeal SCC may benefit from new treatment regimens and modalities such as de-escalation radiotherapy and immunotherapy.^{9,10,146} In our cohort of HR-HPV positive periorbital lesions, the extracted clinical outcome information for patients was insufficient to draw definitive conclusions regarding future management. Nevertheless, OSSN in general and irrespective of HR-HPV status, carries a favorable prognosis in comparison with tumors of other sites, and many cases are treated successfully with local surgical excision with adjuvant cryotherapy or topical chemotherapy. Recurrence is possible (as high as one-third of cases in a series of 61 patients), but an overall recurrence rate of 12.9% has been reported as more representative in for OSSN.²⁸⁶

Distant metastases to peri-auricular or cervical lymph nodes are exceedingly uncommon. In the current study, local recurrence was not identified in any of our HPV-positive OSSN with available clinical information; however, distant metastasis to a periparotid lymph node was observed in one case of I-SCC. Most OSSN arise from the limbus (corneo-conjunctival junction).^{224,304} In our cohort of HR-HPV positive OSSN, most also arose from the limbic corneo-conjunctival TZ. Pseudoepitheliomatous hyperplasias contrast by usually not arising at the limbus but rather in any of the various quadrants of the epithelial surface away from this zone. The limbus is known to contain a unique population of basal stem cells, whose function is to replace the aged, damaged and diseased corneal cells, thereby maintaining corneal health and transparency.^{287,288,305}

Curiously, while the peripheral cornea is involved by dysplastic epithelium but rarely the central zone, there is a tendency for centrifugal spread toward the conjunctiva.²⁸⁹ It is believed that the limbal BSC are vulnerable to mutational events induced by UVR or could possibly be prone to infection by viruses.²⁸⁹ Given that the majority of HPV-induced squamous lesions of the uterine cervix occur at the ecto-endocervical transition zone, it is tempting to speculate that the corneo-conjunctival transition zone might bear some analogy to the latter.



It has been proposed that HPV infection usually occurs in areas of damaged epithelium, and patients with conjunctivitis or dacryocystitis may be more vulnerable to HPV infection.²⁷ Precisely how HR-HPV initially infects the periocular tissues is currently not well understood. A proposed route of infection is autoinoculation.²¹² It is presumed that patients with genital HR-HPV infection may transfer the virus to the conjunctiva, by hand-to-eye contact or through sexual activity that would expose the periocular tissues to HPV. The virus might then reach the lacrimal sac epithelium through the tear flow. The drainage of tears from the conjunctival sac into the lacrimal sac represents a potential concentration mechanism for HR-HPV, and may contribute to the enhanced proportion of squamous cell carcinomas in the sac. An alternate route of periocular infection is vertical maternal transmission (by passage of the fetus through the birth

canal), with HPV being latent during early life followed by reactivation of the virus in adult life.²¹²HR-HPV might also reach the lacrimal sac mucosa through the lacrimal duct which opens into the nose; 21% of sinonasal carcinomas are reported to be HR- HPV positive. Given that significant proportion of periocular cancers and precancers are associated with HR-HPV, the possibility exists that current efforts to vaccinate children against HR-HPV might also have an impact on the incidence of these periocular lesions.

In contrast to OSSN, which are often detected in the very early pre-invasive stages, most LSSCC are locally invasive at the time of diagnosis.³⁰⁶ The treatment of primary LSSCC often involves wide surgical excision, followed by adjuvant radio and/or chemotherapy.³⁰⁶ In our cohort, 62.5% of HR-HPV positive LSSCC with clinical follow up were locally invasive at the time of diagnosis and were managed by radical surgical procedures including orbital exenteration and maxillectomy. For lacrimal sac carcinomas, the reported survival rates range from 38% to 93% in larger series.³⁰⁷⁻³⁰⁹ A recent study reported a survival rate of 80% with a median follow up period of 27 months; however, it did not examine HR-HPV as a co-factor.³⁰⁶ Among patients in our cohort with HR-HPV positive LSSCC with available clinical follow-up, all were alive after a mean follow-up of 5 years.



4.2 Sebaceous Carcinomas

Previous studies evaluating a potential role for HR-HPV in periocular SC have resorted to various limited methodologies of disparate sensitivity and specificity, while some were limited by the size of the patient cohorts (Table 1.10)^{300,303} One study³⁰¹ identified a single HPV-16 positive instance out of 24 tumors using DNA PCR alone, which may have been a false positive. A similar result was discovered in the current study. The only investigation that identified a significant percentage of HPV positivity in periocular SC was performed on a Japanese population of 24 patients using DNA ISH alone.²⁸ This approach revealed 61.9% HPV positivity overall and 57.1% HR-HPV positivity. The authors stated²⁸ that the “positive signal in the nucleus was observed not only in the cancer cells, but also in the cells of surrounding normal

sebaceous glands and epidermis...we could not determine whether HPV infections were likely to be causative or not," clearly suggesting difficulty in interpreting test results in the setting of high background staining.

Most current methods (Table 1.10) utilized in pathologic studies have depended upon detection of HPV DNA in tissue sections, either by direct hybridization or based on amplification after extraction. Traditional ISH pairs a labelled probe with complementary bases to HPV DNA and, in that way, is the only method to demonstrate the precise localization of the viral genome in an individual tumor cell. Sensitivity of DNA ISH is limited, however, due to difficulty in detecting minute amounts of DNA without amplification. Additionally, the presence of HPV DNA is not synonymous with transcriptional activity. PCR has been regarded as the most sensitive technique of DNA detection. This is the consequence of its ability to amplify minute quantities of DNA, but unfortunately it is less specific because the DNA present cannot be confirmed to be isolated from the tumor cells, normal surrounding tissue, or from an outside contaminant. The current study utilized a novel method of RNA ISH to detect transcriptionally active HR-HPV.³¹⁰ This method includes the ability to amplify minute quantities of HPV RNA and has been shown to be more sensitive but equally specific for HR-HPV detection in comparison with standard DNA ISH. RNA ISH is also easier to interpret due to a brighter, cleaner signal.³¹⁰ DNA ISH may show high non-specific background staining, which as mentioned above, is the probable explanation for the result in a prior study demonstrating HPV positivity in SC.²⁸

Two viral genes, E6 and E7, have been shown to be largely responsible for HPV related carcinogenesis when incorporated into the DNA of various human tissues. The two oncoproteins E6 and E7, when expressed, inactivate the functions of the well-known human tumor suppressor genes p53 and pRb, respectively.^{57,183} E7 forms a complex with the pRb protein causing its inactivation and degradation.⁵⁷ p16 is a tumor suppressor gene related to cell cycle senescence in the pRb pathway and is therefore not expressed in many normal proliferating tissues. Interpretation of p16 staining results depend on the

particular tissue and tumor being studied. For example, in cutaneous nevi, p16 positivity is reassuring and p16 expression is lost in melanoma.³¹¹ On the other hand, in many HPV-induced mucosal tumors p16 expression implies neoplastic transformation. p16 inactivating mutations have been found in several human cancers. It therefore seems paradoxical that p16 would be overexpressed in HPV related cervical, anorectal and oropharyngeal cancers (i.e, that a tumor suppressor gene would be overexpressed in a malignant neoplasm).³¹² The explanation lies in the fact that the p16 gene is located upstream of the pRb gene in the p16/CDK/pRb pathway and its expression is thought to be regulated via a negative feedback control mechanism by pRb protein levels.³¹³ Binding of E7 leading to degradation of the pRb protein will result in increased p16 expression, which can be used as a surrogate marker of HPV infection.³¹²

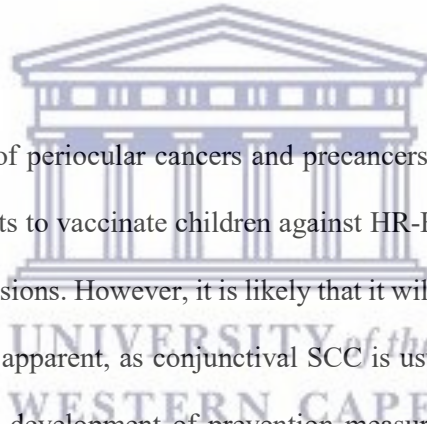
Theoretically, other downstream perturbations of the cell cycle pathway described above could also lead to p16 expression without obligating the presence of HPV. Aberrant growth signals and increased cell division may also induce p16 expression. As described in the current new cohort of patients with periocular SC, p16 has been demonstrated to be immunohistochemically positive in other tumors (e.g., SCC of the skin) without evidence of HPV infection.³¹⁴

Two earlier studies of periocular SC investigated both p16 immunoreactivity and tumor HPV status, although the threshold for p16 positivity was not as rigorous as the one used in the current study.^{300,301} Both found no evidence of HPV, despite a high rate of p16 positivity in one study. A more recent analysis of periocular SC which did not study the role of HPV, reported greater than 90% p16 positivity, similar to our results,⁴¹ a higher positive percentage than that found with immunohistochemical staining for p53. These results, combined with those of the present study, suggest that the immunohistochemical identification of p16 should be added to the current diagnostic armamentarium^{41,298,315,316} for identifying subtle single cell intraepithelial (pagetoid) spread of tumor or small units of microinvasive disease, especially in small conjunctival map biopsies.

CHAPTER 5

Conclusion and Future Directions

In conclusion, our study supports a role for HR-HPV in the development of a subset of OSSN and LSSCC with predominantly non-keratinizing histomorphology. Based upon our findings using p16 immunohistochemistry coupled with DNA ISH and PCR, the lacrimal sac and conjunctiva appear to constitute the second and third most common head and neck subsites for HR-HPV positive squamous neoplasia.



Given that significant proportion of periocular cancers and precancers are associated with HR-HPV, the possibility exists that current efforts to vaccinate children against HR-HPV might also have an impact on the incidence of these periocular lesions. However, it is likely that it will be many years before the benefits of a vaccination program become apparent, as conjunctival SCC is usually a disease of the older adults. This emphasizes the need for the development of prevention measures to control or prevent recurrent disease, particularly for LSSCC, which often exhibit an aggressive local behaviour.

The emergence of HPV-positive OPSCC has opened up new treatment possibilities. Evasion of antitumor immunity by HNSCC occurs through high expression of PDL-1 and/or tumour immune infiltration by PD-1 positive T lymphocytes. It has been reported that tumor infiltration by PD-1 positive, CD-8 positive and PD-1 positive and CD4-positive lymphocytes is more common among HPV-positive than HPV-negative OPSCC. It would be interesting to study the expression of the inhibitory receptor PD-1 and its ligands in HPV-positive OSSN and LSSCC, since these tumours may potentially respond to anti-PD-1 or PD-L1 agents, which have demonstrated marked clinical efficacy in patients with HPV-positive OPSCC.

Proving HPV linkage to periocular SC or any other tumor requires fastidious techniques with the greatest sensitivity and specificity. These methods have failed to establish a role for HR-HPV in the tumorigenesis of SC. Furthermore, unlike the situation in some tumors of other anatomic sites, p16 is not a reliable indicator of the presence of HPV in periocular SC. It nonetheless has been shown to be a superior diagnostic biomarker for the light microscopic detection of single neoplastic cells.



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REFERENCES

1. LYON F. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans Report of the Advisory Group to Recommend Priorities for IARC Monographs during 2015 – 2019. In: *Some Industrial Chemicals*. Vol 60. ; 1994:389-433.
2. Gillison ML, Koch WM, Capone RB, et al. Evidence for a Causal Association Between Human Papillomavirus and a Subset of Head and Neck Cancers. *JNCI*. 2018;92(9):709-720.
3. Marur S, Souza GD, Westra WH, Forastiere AA. HPV-associated head and neck cancer : a virus-related cancer epidemic. *Lancet Oncol*. 2010;11(8):781-789. doi:10.1016/S1470-2045(10)70017-6
4. Adelstein DJ, Ridge JA, Gillison ML, et al. Monograph Head and Neck Squamous Cell Cancer and the Human Papillomavirus: Summary of a National Cancer Institute State of the Science Meeting. *HNSCC HPV*. 2009;31:1393-1422. doi:10.1002/hed
5. Gillison ML, Chaturvedi AK, Anderson WF, Fakhry C. Epidemiology of human papillomavirus-positive head and neck squamous cell carcinoma. *J Clin Oncol*. 2015;33(29):3235-3242. doi:10.1200/JCO.2015.61.6995
6. Weber R, Rosenthal DI, Nguyen-tân PF, et al. Human Papillomavirus and Survival of Patients with Oropharyngeal Cancer. *N Engl J Med*. 2010;363:24-35.
7. Fakhry C, Westra WH, Li S, et al. Improved Survival of Patients With Human Papillomavirus – Positive Head and Neck Squamous Cell Carcinoma in a Prospective Clinical Trial. *JNCI*. 2008:261-269. doi:10.1093/jnci/djn011
8. Ragin CCR, Taioli E. Survival of squamous cell carcinoma of the head and neck in relation to human papillomavirus infection : Review and meta-analysis. *Int J Cancer*. 2007;121:1813-1820. doi:10.1002/ijc.22851
9. Kimple RJ, Smith MA, Blitzer GC, et al. Enhanced Radiation Sensitivity in HPV-Positive Head and Neck

- Cancer. *AACR*. 2013;73(15):4791-4800. doi:10.1158/0008-5472.CAN-13-0587
10. Lassen P, Eriksen JG, Krogdahl A, et al. The influence of HPV-associated p16-expression on accelerated fractionated radiotherapy in head and neck cancer: Evaluation of the randomised. *Radiother Oncol*. 2011;100(1):49-55. doi:10.1016/j.radonc.2011.02.010
 11. Lyford-pike S, Peng S, Young GD, et al. Evidence for a Role of the PD-1 : PD-L1 Pathway in Immune Resistance of HPV-Associated Head and Neck Squamous Cell Carcinoma. *AACR*. 2013;73(6):1733-1742. doi:10.1158/0008-5472.CAN-12-2384
 12. Kim HS, Lee JY, Park K, et al. Association between PD-L1 and HPV Status and the Prognostic Value of PD-L1 in Oropharyngeal Squamous Cell Carcinoma. *KCA*. 2016;48(2):527-536.
 13. Justin A. Bishop, M.D., Theresa W. Guo, M.D., David F. Smith, M.D., Ph.D., Hao Wang, Ph.D., Takenori Ogawa, M.D., Ph.D., Sara I. Pai, M.D., Ph.D. and WHW, M.D. Human Papillomavirus-Related Carcinomas of the Sinonasal Tract. *Am J Surg Pathol*. 2013;37(2):185-192. doi:10.1097/PAS.0b013e3182698673.Human
 14. Castellsague X, Alemany L, Holzinger D et al. Estimation of the HPV etiological fraction in over 4,000 head and neck cancers worldwide. In: *International Human Papillomavirus Confer- Ence*. Seattle, WA.
 15. Jr JSL, Ukpo OC, Ma X, et al. Transcriptionally-active high-risk human papillomavirus is rare in oral cavity and laryngeal/hypopharyngeal squamous cell carcinomas – a tissue microarray study utilizing E6/E7 mRNA in situ hybridization. *Histopathology*. 2012;60:982-991. doi:10.1111/j.1365-2559.2011.04169.x
 16. McDonnell JM, McDonnell PJ, Yu Y. Human Papillomavirus DNA in Tissues and Ocular Surface Swabs of Patients with Conjunctival Epithelial Neoplasia. *IOVS*. 1992;33(1):184-189.
 17. McDonnell JM, Mayr AJ MW. DNA of human papillomavirus type 16 in dysplastic and malignant lesions of the conjunctiva and cornea. *N Engl J Med*. 1989;320:1442-1446.
 18. Karcioğlu ZA, Issa TM. Human papilloma virus in neoplastic and non-neoplastic conditions of the external eye. *Br J Ophthalmol*. 1997;81:595-598.

19. Lauer SA, Malter JS MJ. Human papillomavirus type 18 in conjunctival intraepithelial neoplasia. *Am J Ophthalmol.* 1990;110:23-27.
20. Tabrizi SN, Mccurrach FE, Drewe RH, Borg AJ, Garland SM, Taylor HR. Original Article Human papillomavirus in corneal and conjunctival carcinoma. *Aust N Z J Opbtbalmolgy.* 1997;25:211-215.
21. Toth J, Karcioğlu ZA, Moshfeghi AA, et al. The Relationship Between Human Papillomavirus and p53 Gene in Conjunctival Squamous Cell Carcinoma. *Cornea.* 2000;19(2):159-162.
22. Nakamura Y, Mashima Y, Kameyama K, Mukai M, Oguchi Y. Detection of human papillomavirus infection in squamous tumours of the conjunctiva and lacrimal sac by immunohistochemistry, in situ hybridisation, and polymerase chain reaction. *Br J Ophthalmol.* 1997;81:308-313.
23. Saegusa M, Takano Y, Hashimura M, Okayasu I, Shiga J. HPV type 16 in conjunctival and junctional papilloma, dysplasia, and squamous cell carcinoma. *J Clin Pathol.* 1995;48(12):1106-1110. doi:10.1136/jcp.48.12.1106
24. Scott IU, Karp CL, Nuovo GJ. Human Papillomavirus 16 and 18 Expression in Conjunctival Intraepithelial Neoplasia. *Elsevier Sci Inc.* 2002:542-547.
25. Salceanu SO, Constantin C, Cijevschi I, Ursu RG1, Grigorovici M IL. Human papillomavirus 52 positive squamous cell carcinoma of the conjunctiva. *Indian J Ophthalmol.* 2015;63:166-169.
26. Woods M, Chow S, Heng B, et al. Detecting Human Papillomavirus in Ocular Surface Diseases. *Invest Ophthalmol Vis Sci.* 2013;54:8069-8078. doi:10.1167/iovs.13-13140
27. Sjo NC, Buchwald C Von, Flamant P, et al. Human papillomavirus : cause of epithelial lacrimal sac neoplasia ? *Acta Ophthalmol Scand.* 2007;85:551-556. doi:10.1111/j.1600-0420.2007.00893.x
28. Ueno NH• MF• YOH. Search for accumulation of p53 protein and detection of human papillomavirus genomes in sebaceous gland carcinoma of the eyelid. *Virchows Arch.* 1994;424:503-509.
29. Eng H, Lin T, Chen S, Wu S, Chen W. Failure to Detect Human Papillomavirus DNA in Malignant Epithelial

- Neoplasms of Conjunctiva by Polymerase. *Am J Clin Pathol.* 2002;117(3):429-436.
30. Guthoff R, Marx A SP. . No evidence for a pathogenic role of human papillomavirus infection in ocular surface squamous neoplasia in Germany. *Curr Eye Res.* 2009;34:666-671.
31. Tuppurainen K, Raninen A, Kosunen O, Kankkunen JP, Kellokoski J, Syrjänen S, Mäntyjärvi M SK. . Squamous cell carcinoma of the conjunctiva. Failure to demonstrate HPV DNA by in situ hybridization and polymerase chain reaction. *Acta Ophthalmol.* 1992;70:248-254.
32. Yu JJ, Fu P, Pink JJ, et al. HPV Infection and EGFR Activation / Alteration in HIV-Infected East African Patients with Conjunctival Carcinoma. *PLoS One.* 2010;5(5):3-8. doi:10.1371/journal.pone.0010477
33. Merz H, Malisius R, Mannweiler S, Zhou R, Hartmann W, Orscheschek K, Moubayed P FA. ImmunoMax. A maximized immunohistochemical method for the retrieval and enhancement of hidden antigens. . *Lab Investig a J Tech methods Pathol.* 73(1):149-156.
34. Koning MNC De, Waddell K, Magyezi J, et al. Genital and cutaneous human papillomavirus (HPV) types in relation to conjunctival squamous cell neoplasia : A case-control study in Uganda. *Infect Agent Cancer.* 2008;3(12):1-9. doi:10.1186/1750-9378-3-12
35. Waddell K, Magyezi J, Bousarghin L, et al. Antibodies against human papillomavirus type 16 (HPV-16) and conjunctival squamous cell neoplasia in Uganda. *Br J Cancer.* 2003;88:2002-2003. doi:10.1038/sj.bjc.6600950
36. Auw-haedrich C, Martin G, Spelsberg H, et al. Expression of p16 in Conjunctival Intraepithelial Neoplasia Does Not Correlate with HPV-Infection. *Open Ophthalmol J.* 2008;2:48-56.
37. Nakamura Y, Mashima Y, Kameyama K, Mukai M, Oguchi Y. Detection of human papillomavirus infection in squamous tumours of the conjunctiva and lacrimal sac by immunohistochemistry, in situ hybridisation, and polymerase chain reaction. *Br J Ophthalmol.* 1997;81:308-313.
38. WHO. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. *IARC Monogr.* 2012;100

B:1-441.

39. Newton R, Ziegler J, Bousarghin L, et al. The epidemiology of conjunctival squamous cell carcinoma in Uganda. *Br J Cancer*. 2002;(87):301-308. doi:10.1038/sj.bjc.6600451
40. Lee GA HL. Incidence of ocular surface epithelial dysplasia in metropolitan Brisbane: a 10-year survey. *Arch Ophthalmol*. 110(4):525-527.
41. Bell WR, Singh K, Rajan A, Charles KD. Expression of p16 and p53 in Intraepithelial Periocular Sebaceous Carcinoma. *Ocul Oncol Pathol*. 2016;2:71-75. doi:10.1159/000439308
42. Kim N, Eun J, Choung H, Joung M, In S. Expression of cell cycle regulatory proteins in eyelid sebaceous gland carcinoma: Low p27 expression predicts poor prognosis q. *Exp Eye Res*. 2014;118:46-52. doi:10.1016/j.exer.2013.10.022
43. Niu Y, Zhou Z, Liu F WH. Expression of P16 protein and Bcl-2 protein in malignant eyelid tumors. *Chin Med J*. 115(1):21-25.
44. McBride AA. Oncogenic human papillomaviruses. *Philos Trans R Soc B Biol Sci*. 2017;372(1732):20160273. doi:10.1098/rstb.2016.0273
45. Gottschling M, Bravo IG, Sanjose S De. The clinical importance of understanding the evolution of papillomaviruses. *Trends Microbiol*. 2010;18(10):432-436. doi:10.1016/j.tim.2010.07.008
46. Mavian C, Labella AM, Castro D, Borrego JJ, Alcami A. Concurrence of Iridovirus, Polyomavirus, and a Unique Member of a New Group of Fish Papillomaviruses in Lymphocystis Disease-Affected. *J Virol*. 2016;90(19):8768-8779. doi:10.1128/JVI.01369-16.Editor
47. Shah SD, Doorbar J, Goldstein RA. Analysis of host-parasite incongruence in papillomavirus evolution using importance sampling. *Mol Biol Evol*. 2010;27(6):1301-1314. doi:10.1093/molbev/msq015
48. Saraiya M, Unger ER, Thompson TD, et al. US assessment of HPV Types in cancers: Implications for current and 9-valent HPV vaccines. *J Natl Cancer Inst*. 2015;107(6). doi:10.1093/jnci/djv086

49. John Doorbar, Nagayasu Egawa, Heather Griffin CK and IM. Human papillomavirus molecular biology and disease association. *Rev Med Virol.* 2015;25(1):2-23. doi:10.1002/rmv
50. Bergvall M, Melendy T, Archambault J. The E1 proteins. *Virology.* 2013;445:35-56. doi:10.1016/j.virol.2013.07.020
51. McBride AA. The Papillomavirus E2 proteins. *Virology.* 2013;445(1-2):57-79. doi:10.1016/j.virol.2013.06.006
52. Wang JW, Roden RBS. L2, the minor capsid protein of papillomavirus. *Virology.* 2013;445(1-2):175-186. doi:10.1016/j.virol.2013.04.017
53. Buck CB, Day PM, Trus BL. The papillomavirus major capsid protein L1. *Virology.* 2013;445(1-2):169-174. doi:10.1016/j.virol.2013.05.038
54. Doorbar J. The E4 protein ; structure, function and patterns of expression. *Virology.* 2013;445(1-2):80-98. doi:10.1016/j.virol.2013.07.008
55. Dimaio D, Petti LM. The E5 proteins. *Virology.* 2013;445:99-114. doi:10.1016/j.virol.2013.05.006
56. Vande SB, Klingelutz AJ. Papillomavirus E6 oncoproteins. *Virology.* 2013;445(1-2):115-137. doi:10.1016/j.virol.2013.04.026
57. Roman A, Munger K. The papillomavirus E7 proteins. *Virology.* 2013;445(1-2):138-168. doi:10.1016/j.virol.2013.04.013
58. Doorslaer K Van, McBride AA. Molecular archeological evidence in support of the repeated loss of a papillomavirus gene. *Sci Rep.* 2016;6:33028. doi:10.1038/srep33028
59. Chen XS, Garcea RL, Goldberg I, Casini G, Harrison SC. Structure of small virus-like particles assembled from the L1 protein of human papillomavirus 16. *Mol Cell.* 2000;5(3):557-567. doi:10.1016/S1097-2765(00)80449-9

60. Chen XS, Casini G, Harrison SC, Garcea RL. Papillomavirus Capsid Protein Expression in Escherichia coli : Purification and Assembly of HPV11 and HPV16 L1. *J Mol Biol.* 2001;307:173-182. doi:10.1006/jmbi.2001.4464
61. Modis Y, Trus BL, Harrison SC. Atomic model of the papillomavirus capsid. *EMBO.* 2002;21(18):4754-4761.
62. Wolf M, Garcea RL, Grigorieff N, Harrison SC. Subunit interactions in bovine papillomavirus. *Proc Natl Acad Sci.* 2010;107(14):6298-6303. doi:10.1073/pnas.0914604107
63. Rubio I, Seitz H, Canali E, et al. The N-terminal region of the human papillomavirus L2 protein contains overlapping binding sites for neutralizing, cross-neutralizing and non-neutralizing antibodies. *Virology.* 2011;409(2):348-359. doi:10.1016/j.virol.2010.10.017
64. Liu WJ, Gissmann L, Sun XY, et al. Sequence close to the N-terminus of L2 protein is displayed on the surface of bovine papillomavirus type 1 virions. *Virology.* 1997;227(2):474-483. doi:10.1006/viro.1996.8348
65. Kines RC, Thompson CD, Lowy DR, Schiller JT, Day PM. The initial steps leading to papillomavirus infection occur on the basement membrane prior to cell surface binding. *Proc Natl Acad Sci.* 2009;106(48):20458-20463. doi:10.1073/pnas.0908502106
66. de Villiers EM. Cross-roads in the classification of papillomaviruses. *Virology.* 2013;445(1-2):2-10. doi:10.1016/j.virol.2013.04.023
67. De Villiers EM, Fauquet C, Broker TR, Bernard HU, Zur Hausen H. Classification of papillomaviruses. *Virology.* 2004;324(1):17-27. doi:10.1016/j.virol.2004.03.033
68. Gillison ML, Alemany L, Snijders PJF, et al. Human Papillomavirus and Diseases of the Upper Airway : Head and Neck Cancer and Respiratory Papillomatosis. *Vaccine.* 2012;30:F34-F54. doi:10.1016/j.vaccine.2012.05.070
69. Syrjänen S. Human papillomavirus infections and oral tumors. *Med Microbiol Immunol.* 2003;192(3):123-128. doi:10.1007/s00430-002-0173-7

70. Gariglio P, Gutiérrez J, Cortés E, Vázquez J. The Role of Retinoid Deficiency and Estrogens as Cofactors in Cervical Cancer. *Arch Med Res*. 2009;40(6):449-465. doi:10.1016/j.arcmed.2009.08.002
71. Roberts JN, Buck CB, Thompson CD, et al. Genital transmission of HPV in a mouse model is potentiated by nonoxynol-9 and inhibited by carrageenan. *Nat Med*. 2007;13(7):857-861. doi:10.1038/nm1598
72. Day PM, Schelhaas M. Concepts of papillomavirus entry into host cells. *Curr Opin Virol*. 2014;4:24-31. doi:10.1016/j.coviro.2013.11.002
73. Woodham AW, Taylor JR, Jimenez AI, et al. Small molecule inhibitors of the annexin A2 heterotetramer prevent human papillomavirus type 16 infection. *J Antimicrob Chemother*. 2015;70:1686-1690. doi:10.1093/jac/dkv045
74. Digiuseppe S, Luszczek W, Keiffer TR, Bienkowska-haba M, Guion LGM. Incoming human papillomavirus type 16 genome resides in a vesicular compartment throughout mitosis. *PNAS*. 2016;113(22):6289-6294. doi:10.1073/pnas.1600638113
75. Pyeon D, Pearce SM, Lank SM, Ahlquist P, Lambert PF. Establishment of Human Papillomavirus Infection Requires Cell Cycle Progression. *PLoS Pathog*. 2009;5(2):1-8. doi:10.1371/journal.ppat.1000318
76. Aydin I, Weber S, Snijder B, et al. Large Scale RNAi Reveals the Requirement of Nuclear Envelope Breakdown for Nuclear Import of Human Papillomaviruses. *PLoS Pathog*. 2014;10(5):1-17. doi:10.1371/journal.ppat.1004162
77. Day PM, Baker CC, Lowy DR, Schiller JT. Establishment of papillomavirus infection is enhanced by promyelocytic leukemia protein (PML) expression. *Proc Natl Acad Sci*. 2004;101(39):14252-14257. doi:10.1073/pnas.0404229101
78. Porter SS, Stepp WH, Stamos JD, McBride AA. Host cell restriction factors that limit transcription and replication of human papillomavirus. *Virus Res*. 2017;231:10-20. doi:10.1016/j.virusres.2016.11.014
79. Ozbun MA. Human Papillomavirus Type 31b Infection of Human Keratinocytes and the Onset of Early

- Transcription. *J Virol.* 2002;76(22):11291-11300. doi:10.1128/JVI.76.22.11291-11300.2002
80. McBride AA. Replication and Partitioning of Papillomavirus Genomes Alison. *Adv Virus Res.* 2011;72:155-205. doi:10.1016/S0065-3527(08)00404-1.Replication
81. Kim K, Lambert PF. E1 Protein of Bovine Papillomavirus 1 Is Not Required for the Maintenance of Viral Plasmid DNA Replication. *Virology.* 2002;293:10-14. doi:10.1006/viro.2001.1305
82. Doorbar J. Molecular biology of human papillomavirus infection and cervical cancer. *Clin Sci.* 2006;110:525-541. doi:10.1042/CS20050369
83. Barrow-laing L, Chen W, Roman A. Low- and high-risk human papillomavirus E7 proteins regulate p130 differently. *Virology.* 2010;400(2):233-239. doi:10.1016/j.virol.2010.01.034
84. Roman A. The human papillomavirus E7 protein shines a spotlight on the pRB family member, p130. *Cell Cycle.* 2006;5(6):567-568. doi:10.4161/cc.5.6.2571
85. Felsani A, Mileo AM, Paggi MG. Retinoblastoma family proteins as key targets of the small DNA virus oncoproteins. *Oncogene.* 2006;25:5277-5285. doi:10.1038/sj.onc.1209621
86. Brehm A, Nielsen SJ, Miska EA, et al. The E7 oncoprotein associates with Mi2 and histone deacetylase activity to promote cell growth. *EMBO J.* 1999;18(9):2449-2458. doi:10.1093/emboj/18.9.2449
87. Nguyen DX, Westbrook TF, Mccance DJ. Human Papillomavirus Type 16 E7 Maintains Elevated Levels of the cdc25A Tyrosine Phosphatase during Deregulation of Cell Cycle Arrest. *J Virol.* 2002;76(2):619-632. doi:10.1128/JVI.76.2.619
88. Mclaughlin-drubin ME, Crum CP, Münger K. Human papillomavirus E7 oncoprotein induces KDM6A and KDM6B histone demethylase expression and causes epigenetic reprogramming. *PNAS.* 2011;108(5):2130-2135. doi:10.1073/pnas.1009933108
89. Holland D, Hoppe-Seyler K, Schuller B, et al. Activation of the enhancer of zeste homologue 2 gene by the human papillomavirus E7 oncoprotein. *Cancer Res.* 2008;68(23):9964-9972. doi:10.1158/0008-5472.CAN-

08-1134

90. Galloway DA, Gewin LC, Myers H, et al. Regulation of telomerase by human papillomaviruses. *Cold Spring Harb Symp Quant Biol.* 2005;70:209-215. doi:10.1101/sqb.2005.70.041
91. Gewin L, Galloway DA. E Box-Dependent Activation of Telomerase by Human Papillomavirus Type 16 E6 Does Not Require Induction of c-myc. *J Virol.* 2001;75(15):7198-7201. doi:10.1128/JVI.75.15.7198-7201.2001
92. Klingelhutz AJ, Foster SA MS. Telomerase activation by the E6 gene product of human papillomavirus type 16. *Nature.* 1996;380:79-82.
93. Javier RT. Cell polarity proteins : common targets for tumorigenic human viruses. *Oncogene.* 2008;27:7031-7046. doi:10.1038/onc.2008.352
94. Culp TD, Cladel NM, Balogh KK, Budgeon LR, Mejia AF, Christensen ND. Papillomavirus Particles Assembled in 293TT Cells Are Infectious In Vivo □. *J Virol.* 2006;80(22):11381-11384. doi:10.1128/JVI.01328-06
95. Kranjec C, Banks L. A Systematic Analysis of Human Papillomavirus (HPV) E6 PDZ Substrates Identifies MAGI-1 as a Major Target of HPV Type 16 (HPV-16) and HPV-18 Whose Loss Accompanies Disruption of Tight Junctions. *J Virol.* 2011;85(4):1757-1764. doi:10.1128/JVI.01756-10
96. Massimi P, Narayan N, Thomas M, et al. Regulation of the hDlg / hScrib / Hugl-1 tumour suppressor complex. *Exp Cell Res.* 2008;314(18):3306-3317. doi:10.1016/j.yexcr.2008.08.016
97. Russo AJ, Magro PG, Hu Z, et al. E2F-1 Overexpression in U2OS Cells Increases Cyclin B1 Levels and cdc2 Kinase Activity and Sensitizes Cells to Antimitotic Agents. *Cancer Res.* 2006;66(14):7253-7261. doi:10.1158/0008-5472.CAN-05-3725
98. Malanchi I, Accardi R, Diehl F, et al. Human Papillomavirus Type 16 E6 Promotes Retinoblastoma Protein Phosphorylation and Cell Cycle Progression Human Papillomavirus Type 16 E6 Promotes Retinoblastoma

- Protein Phosphorylation and Cell Cycle Progression. *Society*. 2004;78(24):13769-13778. doi:10.1128/JVI.78.24.13769
99. Nicolaides L, Davy C, Raj K, Kranjec C, Banks L, Doorbar J. Stabilization of HPV16 E6 protein by PDZ proteins, and potential implications for genome maintenance. *Virology*. 2011;414(2):137-145. doi:10.1016/j.virol.2011.03.017
100. Massimi P, Narayan N, Cuenda A, Banks L. Phosphorylation of the discs large tumour suppressor protein controls its membrane localisation and enhances its susceptibility to HPV E6-induced degradation. *Oncogene*. 2006;25:4276-4285. doi:10.1038/sj.onc.1209457
101. Lee C, Laimins LA. Role of the PDZ domain-binding motif of the oncoprotein E6 in the pathogenesis of human papillomavirus type 31. *J Virol*. 2004;78(22):12366-12377. doi:10.1128/JVI.78.22.12366
102. Tummers B, van der Burg SH. High-risk human papillomavirus targets crossroads in immune signaling. *Viruses*. 2015;7(5):2485-2506. doi:10.3390/v7052485
103. Krawczyk E, Suprynowicz FA, Liu X, et al. A cooperative interaction between the human papillomavirus E5 and E6 oncoproteins. *Am J Pathol*. 2008;173(3):682-688. doi:10.2353/ajpath.2008.080280
104. McIntosh PB, Martin SR, Jackson DJ, et al. Structural Analysis Reveals an Amyloid Form of the Human Papillomavirus Type 16 E1 E4 Protein and Provides a Molecular Basis for Its Accumulation. *J Virol*. 2008;82(16):8196-8203. doi:10.1128/JVI.00509-08
105. Wang Q, Griffin H, Southern S, et al. Functional Analysis of the Human Papillomavirus Type 16 E1 E4 Protein Provides a Mechanism for In Vivo and In Vitro Keratin Filament Reorganization. *J Virol*. 2004;78(2):821-833. doi:10.1128/JVI.78.2.821
106. Brown DR, Kitchin D, Qadadri B, Neptune N, Batteiger T, Ermel A. The human papillomavirus type 11 E1 E4 protein is a transglutaminase 3 substrate and induces abnormalities of the cornified cell envelope. *Virology*.

2006;345:290-298. doi:10.1016/j.virol.2005.09.048

107. Holmgren SC, Patterson N a, Ozburn M a, Lambert PF. The Minor Capsid Protein L2 Contributes to Two Steps in the Human Papillomavirus Type 31 Life Cycle The Minor Capsid Protein L2 Contributes to Two Steps in the Human Papillomavirus Type 31 Life Cycle. *J Virol.* 2005;79(7):3938-3948. doi:10.1128/JVI.79.7.3938
108. Day PM, Roden RB, Lowy DR, Schiller JT. The papillomavirus minor capsid protein, L2, induces localization of the major capsid protein, L1, and the viral transcription/replication protein, E2, to PML oncogenic domains. *J Virol.* 1998;72(1):142-150.
109. Buck CB, Thompson CD, Pang Y-YS, Lowy DR, Schiller JT. Maturation of Papillomavirus Capsids. *J Virol.* 2005;79(5):2839-2846. doi:10.1128/JVI.79.5.2839-2846.2005
110. Finnen RL, Erickson KD, Chen XS, Garcea RL, Finnen L. Interactions between Papillomavirus L1 and L2 Capsid Proteins Interactions between Papillomavirus L1 and L2 Capsid Proteins. *Society.* 2003;77(8):4818-4826. doi:10.1128/JVI.77.8.4818
111. Plummer M, Martel C De, Vignat J, Ferlay J, Bray F, Franceschi S. Global burden of cancers attributable to infections in 2012 : a synthetic analysis. *Lancet Glob Heal.* 2015;4(9):e609-e616. doi:10.1016/S2214-109X(16)30143-7
112. Hausen H. Papillomaviruses in the causation of human cancers — a brief historical account. *Virology.* 2009;384(2):260-265. doi:10.1016/j.virol.2008.11.046
113. Cancer IA for R on. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, in Human Papillomaviruses.* Lyon, France: World Health Organization; 2007.
114. Goon P, Sonnex C, Jani P, Stanley M, Sudho H. Recurrent respiratory papillomatosis : an overview of current thinking and treatment. *Eur Arch Otorhinolaryngol.* 2008;265:147-151. doi:10.1007/s00405-007-0546-z
115. Gillison ML, Shah K V. Human papillomavirus – associated head and neck squamous cell carcinoma :

- mounting evidence for an etiologic role for human papillomavirus in a subset of head and neck cancers. *Curr Opin Oncol.* 2001;13:183-188.
116. Chaturvedi AK, Anderson WF, Lortet-tieulent J, et al. Worldwide Trends in Incidence Rates for Oral Cavity and Oropharyngeal Cancers. *J Clin Oncol.* 2013;31(36):4550-4559. doi:10.1200/JCO.2013.50.3870
117. Jemal A, Edgar P, Dorell C, et al. Annual Report to the Nation on the Status of Cancer, 1975–2009, Featuring the Burden and Trends in Human Papillomavirus (HPV)–Associated Cancers and HPV Vaccination Coverage Levels. *JNCI.* 2018;(15):175-201. doi:10.1093/jnci/djs491
118. Bhosale PG, Pandey M, Desai RS, Patil A. Low prevalence of transcriptionally active human papilloma virus in Indian patients with HNSCC and leukoplakia. *Oral Surgery, Oral Med Oral Pathol Oral Radiol.* 2016;122(5):609-618.e7. doi:10.1016/j.oooo.2016.06.006
119. Viens LJ, Henley SJ, Watson M, Markowitz LE, Thomas CC. Human Papillomavirus – Associated Cancers — United States, 2008 – 2012. *MMWR Morb Mortal Wkly.* 2016;65(26):661-666.
120. Lewis JS, Beadle B, Bishop JA, et al. Human Papillomavirus Testing in Head and Neck Carcinomas: Guideline From the College of American Pathologists. *Arch Pathol Lab Med.* 2017:arpa.2017-0286-CP. doi:10.5858/arpa.2017-0286-CP
121. Martel C De, Plummer M, Vignat J, Franceschi S. Worldwide burden of cancer attributable to HPV by site, country and HPV type. *Int J Cancer.* 2017;141:664-670. doi:10.1002/ijc.30716
122. Castellsagué X, Alemany L, Quer M, et al. HPV Involvement in Head and Neck Cancers : Comprehensive Assessment of Biomarkers in 3680 Patients. *JNCI J Natl Cancer Inst.* 2016;108(6):1-12. doi:10.1093/jnci/djv403
123. Gondim DD, Haynes W, Wang X, Chernock RD, El-mofty SK, Lewis JS. Histologic Typing in Oropharyngeal Squamous Cell Carcinoma A 4-Year Prospective Practice Study With p16 and High-Risk HPV mRNA Testing Correlation. *Am J Surg Pathol.* 2016;40(8):1117-1124.

124. Sullivan BO, Hui S, Perez-ordonez B, et al. Outcomes of HPV-related oropharyngeal cancer patients treated by radiotherapy alone using altered fractionation q. *Radiother Oncol.* 2012;103(1):49-56. doi:10.1016/j.radonc.2012.02.009
125. Services UD of H and H. *Trends in Hospital Utilization.*; 1989.
126. Fakhry C, Andersen KK, Christensen J, Agrawal N, Eisele DW. The Impact of Tonsillectomy upon the Risk of Oropharyngeal Carcinoma Diagnosis and Prognosis in the Danish Cancer Registry. *AACR.* 2015:583-590. doi:10.1158/1940-6207.CAPR-15-0101
127. Chaturvedi AK. Tonsillectomy and Risk of Oropharyngeal Cancer : Implications for Research and Prevention. *AACR.* 2015:577-580. doi:10.1158/1940-6207.CAPR-15-0135
128. Ng M, Freeman MK, Fleming TD, et al. Smoking Prevalence and Cigarette Consumption in 187 Countries, 1980-2012. *JAMA Intern Med.* 2014;98121(2):183-192. doi:10.1001/jama.2013.284692
129. Chaturvedi AK, Engels EA, Pfeiffer RM, et al. Human Papillomavirus and Rising Oropharyngeal Cancer Incidence in the United States. *J Clin Oncol.* 2011;29(32):4294-4301. doi:10.1200/JCO.2011.36.4596
130. D'Souza G, Kreimer A, Viscidi R, et al. Case-control study of human papillomavirus and oropharyngeal cancer. *N Engl J Med.* 2007;356(19):1944-1956.
131. Gillison ML, D'Souza G, Westra W, et al. Distinct risk factor profiles for human papillomavirus type 16-positive and human papillomavirus type 16-negative head and neck cancers. *J Natl Cancer Inst.* 2008;100(6):407-420. doi:10.1093/jnci/djn025
132. Singhi AD, Westra WH. Comparison of Human Papillomavirus In Situ Hybridization and p16 Immunohistochemistry in the Detection of Human Papillomavirus- Associated Head and Neck Cancer Based on a Prospective Clinical Experience. *Cancer.* 2010:2166-2173. doi:10.1002/cncr.25033
133. Heck JE, Berthiller J, Vaccarella S, et al. Sexual behaviours and the risk of head and neck cancers : a pooled analysis in the International Head and Neck Cancer Epidemiology (INHANCE) consortium. *Int J Epidemiol.*

- 2010;39:166-181. doi:10.1093/ije/dyp350
134. Kahle L, Graubard BI, Chaturvedi AK. Prevalence of Oral HPV Infection in the United States, 2009-2010. *JAMA*. 2012;307(7):693-703. doi:10.1001/jama.2012.101
135. Mbulawa ZZA, Johnson LF, Marais DJ, Coetzee D, Williamson A. Risk factors for oral human papillomavirus in heterosexual couples in an African setting. *J Infect*. 2014;68(2):185-189. doi:10.1016/j.jinf.2013.10.012
136. Vogt SL, Gravitt PE, Martinson NA, Hoffmann J, Souza GD. Concordant oral-genital HPV infection in South Africa couples : evidence for transmission. *Front Oncol*. 2013;3(303):1-7. doi:10.3389/fonc.2013.00303
137. Kreimer R, Campbell CMP, Goodman MT, Giuliano AR, Nyitray AG. International Journal of Cancer papillomavirus infection natural history, transmission and anatomic site of infection. *Int J Cancer*. 2015;136:2752-2760. doi:10.1002/ije.29082
138. Ho GYF, Studentsov Y, Hall CB, et al. Risk Factors for Subsequent Cervicovaginal Human Papillomavirus (HPV) Infection and the Protective Role of Antibodies to HPV-16 Virus-Like Particles. *J Infect Dis*. 2002;186:737-742.
139. Safaean M, Porras C, Schiffman M, et al. Epidemiological Study of Anti-HPV16 / 18 Seropositivity and Subsequent Risk of HPV16 and -18 Infections. *JNCI*. 2010;102(21):1653-1662. doi:10.1093/jnci/djq384
140. Wilson L, Pawlita M, Castle PE, et al. Seroprevalence of 8 Oncogenic Human Papillomavirus Genotypes and Acquired Immunity Against Reinfection. *JID*. 2014;210:448-455. doi:10.1093/infdis/jiu104
141. Pickard RKL, Xiao W, Broutian TR. The Prevalence and Incidence of Oral Human Papillomavirus Infection Among Young Men and Women, Aged 18 – 30 Years. *Sex Transm Dis*. 2012;39(7):559-566. doi:10.1097/OLQ.0b013e31824f1c65
142. Anantharaman D, Muller DC, Lagiou P, et al. Combined effects of smoking and HPV16 in oropharyngeal cancer. *Int J Epidemiol*. 2016;45(3):752-761. doi:10.1093/ije/dyw069
143. Chaturvedi AK, Souza GD, Gillison ML, Katki HA. Burden of HPV-positive oropharynx cancers among ever

- and never smokers in the U . S . population. *Oral Oncol.* 2016;60:61-67.
doi:10.1016/j.oraloncology.2016.06.006
144. Rietbergen MM, Braakhuis BJM, Moukhtari N, et al. No evidence for active human papillomavirus (HPV) in fields surrounding HPV-positive oropharyngeal tumors. *J Oral Pathol Med.* 2014;43:137-142.
doi:10.1111/jop.12123
145. Cantrell SC, Peck BW, Li G, Wei Q, Sturgis EM, Ginsberg LE. Differences in Imaging Characteristics of HPV-Positive and HPV-Negative Oropharyngeal Cancers : *AJNR Am J Neuroradio.* 2013;34:2005-2009.
146. Rieckmann T, Tribius S, Grob TJ, et al. HNSCC cell lines positive for HPV and p16 possess higher cellular radiosensitivity due to an impaired DSB repair capacity. *Radiother Oncol.* 2013;107(2):242-246.
doi:10.1016/j.radonc.2013.03.013
147. Weaver AN, Cooper TS, Rodriguez M, et al. DNA double strand break repair defect and sensitivity to poly ADP- ribose polymerase (PARP) inhibition in human papillomavirus 16-positive head and neck squamous cell carcinoma. *Oncotarget.* 2015;6(29):26995-27007.
148. Posner MR, Lorch JH, Goloubeva O, et al. Survival and human papillomavirus in oropharynx cancer in TAX 324: a subset analysis from an international phase III trial. *Ann Oncol.* 2011;22:1071-1077.
doi:10.1093/annonc/mdr006
149. Rischin D, Young RJ, Fisher R, et al. Prognostic Significance of p16 INK4A and Human Papillomavirus in Patients With Oropharyngeal Cancer Treated on TROG 02 . 02 Phase III Trial. *J Clin Oncol.* 2010;28(27):4142-4148. doi:10.1200/JCO.2010.29.2904
150. Fakhry C, Zhang Q, Nguyen-tan PF, et al. Human Papillomavirus and Overall Survival After Progression of Oropharyngeal Squamous Cell Carcinoma. *J Clin Oncol.* 2014;32(30):3365-3373.
doi:10.1200/JCO.2014.55.1937
151. Sullivan BO, Huang SH, Su J, et al. Development and validation of a staging system for HPV-related oropharyngeal cancer by the International Collaboration on Oropharyngeal cancer Network for Staging (

- ICON-S): a multicentre cohort study. *Lancet Oncol.* 2016;17(4):440-451. doi:10.1016/S1470-2045(15)00560-4
152. Brierley JD, Gospodarowicz MK WC. *TNM Classification of Malignant Tumours*. 8th ed. (Brierley JD, Gospodarowicz MK, Wittekind C, eds.). Wiley Blackwell; 2017.
153. Wuthrick EJ, Zhang Q, Machtay M, et al. Institutional Clinical Trial Accrual Volume and Survival of Patients With Head and Neck Cancer. *J Clin Oncol.* 2015;33(2):156-164. doi:10.1200/JCO.2014.56.5218
154. Sullivan BO, Huang SH, Siu LL, Waldron J, Zhao H, Perez-ordonez B. Deintensification Candidate Subgroups in Human Papillomavirus-Related Oropharyngeal Cancer According to Minimal Risk of Distant Metastasis. *J Clin Oncol.* 2013;31(5):543-550. doi:10.1200/JCO.2012.44.0164
155. Alemany L, Gillison ML, Mes R. Human papillomavirus-related oropharyngeal cancer. *Ann Oncol.* 2017;28(10):2386-2398. doi:10.1093/annonc/mdx304
156. Mills SE. *Histology for Pathologist*. 4th ed. Lippincott Williams & Wilkins; 2012.
157. Perry ME, JonesMM M. Structure of the crypt epithelium in human palatine tonsils. *Acta Oto-Laryngologica Suppl.* 1988;454:53-59.
158. Agrawal N, Frederick MJ, Pickering CR, et al. Exome Sequencing of Head and Neck Squamous Cell Carcinoma Reveals Inactivating Mutations in NOTCH1. *Science (80-)*. 2011;333(6046):1154-1157. doi:10.1126/science.1206923.Exome
159. Fallai C, Perrone F, Licitra L, et al. Oropharyngeal Squamous Cell Carcinoma Treated With Radiotherapy or Radiochemotherapy: Prognostic Role of TP53 and HPV Status. *Int J Radiat Oncol Biol Phys.* 2009;75(4):1053-1059. doi:10.1016/j.ijrobp.2008.12.088
160. Cullen AP, Reid R, Campion M, Lorinczl AT. Analysis of the Physical State of Different Human Papillomavirus DNAs in Intraepithelial and Invasive Cervical Neoplasm. *J Virol.* 1991;65(2):606-612.
161. Akagi K, Li J, Broutian TR, et al. Genome-wide analysis of HPV integration in human cancers reveals

- recurrent, focal genomic instability. *Genome Res.* 2014;24(2):185-199. doi:10.1101/gr.164806.113.24
162. Vencalek O, Lukesova E, Klozar J, Tachezy R. IJC International Journal of Cancer Analysis of the integration of human papillomaviruses in head and neck tumours in relation to patients' prognosis. *Int J Cancer.* 2015;138:386-395. doi:10.1002/ijc.29712
163. Lim MY, Dahlstrom KR, Sturgis EM, Li G. Human papillomavirus integration pattern and demographic, clinical, and survival characteristics of patients with oropharyngeal squamous cell carcinoma. *Head Neck.* 2016;38(8):1139-1144. doi:10.1002/HED
164. Giuliano AR, Lee J, Fulp W, et al. Incidence and clearance of genital human papillomavirus infection in men (HIM): a cohort study. *Lancet.* 2011;377(9769):932-940. doi:10.1016/S0140-6736(10)62342-2
165. Jon Mork, M.D., A.Kathrine L IE, M.D., Eystein Glattre, M.D., Göran Hallmans, M.D., Egl Jellum, P H .D., Pentti Koskela, Ph .D., Bjørn Møller, M.S C ., Eero Pukkala, Ph .D., John T. Schiller, P h .D., Linda Youngman, Ph .D., Matti Lehtinen, M. M. Human Papillomavirus Infection as a Risk Factor for Squamous Cell Carcinoma of the Head and Neck. *N Engl J Med.* 2001;344(15):1125-1131.
166. Vu HL, Sikora AG, Fu S, Kao J. HPV-induced oropharyngeal cancer, immune response and response to therapy. *Cancer Lett.* 2010;288(2):149-155. doi:10.1016/j.canlet.2009.06.026
167. Kumar B, Carey TE, Wolf GT. Correlation of Cellular Immunity With Human Papillomavirus 16 Status and Outcome in Patients With Advanced Oropharyngeal Cancer. *JAMA Intern Med.* 2010;136(12):1267-1273.
168. Albers A, Abe K, Hunt J, et al. Antitumor Activity of Human Papillomavirus Type 16 E7 – Specific T Cells against Virally Infected Squamous Cell Carcinoma of the Head and Neck. *Cancer Res.* 2005;65(23):11146-11156. doi:10.1158/0008-5472.CAN-05-0772
169. Ward MJ, Thirdborough SM, Mellows T, et al. Tumour-infiltrating lymphocytes predict for outcome in HPV-positive oropharyngeal cancer. *Br J Cancer.* 2013;110(2):489-500. doi:10.1038/bjc.2013.639
170. Jie H, Schuler PJ, Lee SC, et al. CTLA-4 β Regulatory T Cells Increased in Cetuximab-Treated Head and

- Neck Cancer Patients Suppress NK Cell Cytotoxicity and Correlate with Poor Prognosis. *AACR*. 2015;75(11):2200-2211. doi:10.1158/0008-5472.CAN-14-2788
171. Lina M, Perri F, Buonaguro L, Ionna F, Buonaguro FM, Caponigro F. HPV-related oropharyngeal cancers : From pathogenesis to new therapeutic approaches. *Cancer Lett*. 2014;351(2):198-205. doi:10.1016/j.canlet.2014.06.003
172. Harrington K, Kasper S, Vokes EE, et al. Nivolumab for Recurrent Squamous-Cell Carcinoma of the Head and Neck. *N Engl J Med*. 2016;375(19):1856-1867. doi:10.1056/NEJMoa1602252
173. Bishop JA, Lewis Jr JS, Rocco JW FW. HPV-related squamous cell carcinoma of the head and neck: an update on testing in routine pathology practice. *In Seminars diagnostic Pathol*. 32(5):344-351.
174. Weinberger P, Kowalski D, Harigopal M, Hospital YH. Molecular Classification Identifies a Subset of Human Papillomavirus- Associated Oropharyngeal Cancers With Favorable Prognosis Molecular Classification Identifies a Subset of Human Papillomavirus – Associated Oropharyngeal Cancers With Favorable Prognosi. *J Clin Oncol*. 2006;24(5):736-747. doi:10.1200/JCO.2004.00.3335
175. Smeets SJ, Hesselink AT, Speel EM, et al. A novel algorithm for reliable detection of human papillomavirus in paraffin embedded head and neck cancer specimen. *Int J Cancer*. 2007;121:2465-2472. doi:10.1002/ijc.22980
176. Viola MM, Michiel WM. Biological Evidence that Human Papollomavirus are Etiologically Involved in a Subgroup of Head and Neck. *Int J Cancer*. 2001;93:232-235.
177. Leemans CR, Braakhuis BJM, Brakenhoff RH. The molecular biology of head and neck cancer. *Nat Publ Gr*. 2010;11(1):9-22. doi:10.1038/nrc2982
178. Stein AP, Saha S, Yu M, Kimple RJ, Lambert PF. Prevalence of Human Papillomavirus in Oropharyngeal Squamous Cell Carcinoma in the United States Across Time. *Chem Res Toxicol*. 2014;27:462-469. doi:10.1021/tx500034c

179. Jordan RC, Lingen MW, Perez-ordonez B, et al. Validation of Methods for Oropharyngeal Cancer HPV Status Determination in US Cooperative Group Trials. *Am J Surg Pathol.* 2012;36(7):945-954.
180. JA Bishop, X-J Ma, H Wang, Y Luo, PB Illeil, S Begum, JM Taube, WM Koch A, Westra W. Detection of transcriptionally active high risk HPV in patients with head and neck squamous cell carcinoma as visualized by a novel E6/E7 mRNA in situ hybridization method. *Am J Surg Pathol.* 2012;36(12):1874-1882. doi:10.1097/PAS.0b013e318265fb2b.Detection
181. Ukpo OC, Flanagan JJ, Ma X, Luo Y, Thorstad WL, Lewis JS. High-Risk Human Papillomavirus E6 / E7 mRNA Detection by a Novel In Situ Hybridization Assay Strongly Correlates With p16 Expression and Patient Outcomes in Oropharyngeal Squamous Cell Carcinoma. *Am J Surg Pathol.* 2011;35(9):1343-1350.
182. Schache AG, Liloglou T, Risk JM, et al. Validation of a novel diagnostic standard in HPV-positive oropharyngeal squamous cell carcinoma. *Br J Cancer.* 2013;108(6):1332-1339. doi:10.1038/bjc.2013.63
183. Moody CA, Laimins LA. Human papillomavirus oncoproteins : pathways to transformation. *Nat Publ Gr.* 2010;10(8):550-560. doi:10.1038/nrc2886
184. Mu K, Baldwin A, Edwards KM, et al. Mechanisms of Human Papillomavirus-Induced Oncogenesis. *J Virol.* 2004;78(21):11451-11460. doi:10.1128/JVI.78.21.11451
185. Sedghizadeh PP, Billington WD, Paxton D, et al. Is p16-positive oropharyngeal squamous cell carcinoma associated with favorable prognosis ? A systematic review and meta-analysis. *Oral Oncol.* 2016;54:15-27. doi:10.1016/j.oraloncology.2016.01.002
186. Chernock RD, Wang X, Gao G, et al. Detection and significance of human cell carcinoma of the larynx. *Mod Pathol.* 2012;26(2):223-231. doi:10.1038/modpathol.2012.159
187. Lingen MW, Xiao W, Schmitt A, et al. Low etiologic fraction for high-risk human papillomavirus in oral cavity squamous cell carcinomas. *Oral Oncol.* 2013;49(1):1-8. doi:10.1016/j.oraloncology.2012.07.002
188. Begum S, Cao D, Gillison M, Zahurak M, Westra WH. Human Cancer Biology Tissue Distribution of Human

- Papillomavirus 16 DNA Integration in Patients with Tonsillar Carcinoma. *Clin Cancer Res.* 2005;11(16):5694-5700. doi:10.1158/1078-0432.CCR-05-0587
189. Hayes DN, Waes C Van, Seiwert TY. Genetic Landscape of Human Papillomavirus – Associated Head and Neck Cancer and Comparison to Tobacco-Related Tumors. *J Clin Oncol.* 2015;33(29):3227-3234. doi:10.1200/JCO.2015.62.1086
190. Prigge E, Arbyn M, Reuschenbach M. Oropharyngeal Squamous Cell Carcinomas : A Systematic Review. *Int J Cancer.* 2016;140:1186-1198. doi:10.1002/ijc.30516
191. Rietbergen MM, Brakenhoff RH, Bloemena E, et al. Human papillomavirus detection and comorbidity : critical issues in selection of patients with oropharyngeal cancer for treatment De-escalation trials. *Ann Oncol.* 2013;24:2740-2745. doi:10.1093/annonc/mdt319
192. Thavaraj S, Stokes A, Guerra E, et al. Evaluation of human papillomavirus testing for squamous cell carcinoma of the tonsil in clinical practice. *J Clin Oncol.* 2011;64:308-312. doi:10.1136/jcp.2010.088450
193. Westra WH. The pathology of HPV-related head and neck cancer: Implications for the diagnostic pathologist. *Semin Diagn Pathol.* 2015;32(1):42-53. doi:10.1053/j.semdp.2015.02.023
194. Alos L, Moyano S, Nadal A, Alobid I. Human Papillomaviruses Are Identified in a Subgroup of Sinonasal Squamous Cell Carcinomas With Favorable Outcome. *Cancer.* 2009;115(12):2701-2709. doi:10.1002/ncr.24309
195. Chaudhary AK, Pandya S, Mehrotra R, Bharti AC, Singh M, Singh M. Comparative study between the Hybrid Capture II test and PCR based assay for the detection of human papillomavirus DNA in oral submucous fibrosis and oral squamous cell carcinoma. *Virol J.* 2010;7(253):1-10.
196. Duncan LD, Winkler M, Carlson ER, Heidel RE, Kang E WD. Immunohistochemistry can be used to detect human papillomavirus in oral cavity squamous cell carcinoma. *J Oral Maxillofac Surg.* 2013;71(8):1367-1785.

197. Decaestecker C, Ernoux-neufcoeur P. Human Papillomavirus DNA Strongly Correlates With a Poorer Prognosis in Oral Cavity Carcinoma. *Laryngoscope*. 2012;122(7):1558-1565. doi:10.1002/lary.23298
198. Elango KJ, Suresh A, Erode EM. Role of Human Papilloma Virus in Oral Tongue Squamous Cell Carcinoma Role of Human Papilloma Virus in Oral Tongue Squamous Cell Carcinoma. *APJCP*. 2011;12(4):889-896.
199. Kaminagakura E, Villa LL, Andreoli MA, et al. High-risk human papillomavirus in oral squamous cell carcinoma of young patients. *Int J Cancer*. 2012;130(8):1726-1732. doi:10.1002/ijc.26185
200. Laco J, Slaninka I, Jirásek M, Čelakovský P, Vošmiková H, Ryška A. High-risk human papillomavirus infection and p16INK4protein expression in laryngeal lesions. *Pathol Res Pract*. 2008;204(8):545-552. doi:10.1016/j.prp.2008.03.001
201. Wendt M, Romanitan M, Anders N. Presence of human papillomaviruses and p16 expression in hypopharyngeal cancer. *Head Neck*. 2014;36(1):107-112. doi:10.1002/HED
202. Larque AB, Hakim S, Ordi J, et al. High-risk human papillomavirus is transcriptionally active in a subset of sinonasal squamous cell carcinomas. *Mod Pathol*. 2014;27(3):343-351. doi:10.1038/modpathol.2013.155
203. Furrer VE, Benitez MB, Furnes M, Lanfranchi HE, Modesti NM. Biopsy vs. superficial scraping: detection of human papillomavirus 6, 11, 16, and 18 in potentially malignant and malignant oral lesions. *J Oral Pathol Med*. 2006;35(6):338-344.
204. Li X, Gao L, Li H, et al. Human Papillomavirus Infection and Laryngeal Cancer Risk : A Systematic Review and Meta- Analysis. *JID*. 2013;207(3):479-488. doi:10.1093/infdis/jis698
205. Syrjänen K, Dds SS. Detection of human papillomavirus in sinonasal carcinoma : systematic review and meta-analysis. *Hum Pathol*. 2013;44(6):983-991. doi:10.1016/j.humpath.2012.08.017
206. Isayeva T, Li Y, Maswahu D, Brandwein-gensler M. Human Papillomavirus in Non-Oropharyngeal Head and Neck Cancers : A Systematic Literature Review. *Head Neck Pathol*. 2012;6(1):104-120. doi:10.1007/s12105-012-0368-1

207. Maxwell JH, Kumar B, Feng FY, et al. HPV-Positive/P16-Positive/EBV-Negative Nasopharyngeal Carcinoma in White North Americans. *Head Neck*. 2010;32(5):562-567. doi:10.1002/hed
208. Stenmark MH, Mchugh JB, Schipper M, et al. Nonendemic HPV-Positive Nasopharyngeal Carcinoma : Association With Poor Prognosis. *Radiat Oncol Biol*. 2014;88(3):580-588. doi:10.1016/j.ijrobp.2013.11.246
209. Singhi AD, Califano J W. High-risk human papillomavirus in nasopharyngeal carcinoma. *Head Neck*. 2012;34(2):213-218.
210. Dogan S, Hedberg ML, Ferris RL, Rath TJ, Assaad AM CS. Human papillomavirus and Epstein–Barr virus in naso- pharyngeal carcinoma in a low-incidence population. *Head Neck*. 2014;36(4):511-516.
211. McDonnell PJ, McDonnell JM, Kessis T, Green WR SK. . Detection of human papillomavirus type 6/11 DNA in conjunctival papillomas by in situ hybridization with radioactive probes. *Hum Pathol*. 18(11):1115-1119.
212. Palazzi MA, Erwenne CM, Villa LL. Detection of human papillomavirus in epithelial lesions of the conjunctiva. *Sao Paulo Med J*. 2000;118(5):125-130. doi:10.1590/S1516-31802000000500003
213. Bailey RN GM. Diagnosis and management of conjunctival papillomas. . *J Am Optom Assoc*. 61(5):405-412.
214. Sjo NC, Buchwald C Von, Cassonnet P, et al. Conjunctival Papilloma : Types and Frequencies in a Large Series. *Br J Ophthalmol*. 2007;91:1014-1015. doi:10.1136/bjo.2006.108811
215. Hughes DS, Powell N, Fiander AN. Will vaccination against human papillomavirus prevent eye disease ? A review of the evidence. *Br J Ophthalmol*. 2007;92:460-465. doi:10.1136/bjo.2007.135038
216. Coroneo M. Ultraviolet Radiation and the Anterior Eye. *Eye Contact Lens*. 2011;0(0):1-11. doi:10.1097/ICL.0b013e318223394e
217. Lawrence W. Hirst, MD, MPH; Roy A. Axelsen, BSc, MD, FRCPA; Ivan Schwab M. Pterygium and Associated Ocular Surface Squamous Neoplasia. *Arch Ophthalmol*. 2009;127(1):31-32.
218. Chui J, Coroneo MT, Tat LT, Crouch R, Wakefield D, Girolamo N Di. Ophthalmic Pterygium A Stem Cell

- Disorder with Premalignant Features. *AJPA*. 2011;178(2):817-827. doi:10.1016/j.ajpath.2010.10.037
219. Di Girolamo N. Association of human papilloma virus with pterygia and ocular-surface squamous neoplasia. *Eye*. 2012;26(2):202-211. doi:10.1038/eye.2011.312
220. Luthra R, Nemesure BB, Wu S, Xie SH, Leske MC. Frequency and Risk Factors for Pterygium in the Barbados Eye Study. *Arch Ophthalmol*. 2001;119:1827-1832.
221. Di N, Chui J, Coroneo MT, Wakefield D. Pathogenesis of pterygia : role of cytokines, growth factors, and matrix metalloproteinases. *Prog Retin Eye Res*. 2004;23:195-228. doi:10.1016/j.preteyeres.2004.02.002
222. Detorakis ET, Drakonaki EE S DA. Molecular genetic alterations and viral presence in ophthalmic pterygium. *Int J Mol Med*. 2000;6(1):35-76.
223. Lee GA, Hirst LW. Ocular Surface Squamous Neoplasia. *Surv Ophthalmol* 39. 1995;39(6):429-450.
224. Erie JC, Campbell RJ LT. Conjunctival and corneal intraepithelial and invasive neoplasia. *Ophthalmology*. 93(2):176-183.
225. Jakobiec FA, Rootman J JJ. Squamous cell carcinoma of conjunctiva. *Clin Ophthalmol Philadelphia, PA Lippincott*. 1998;2:20-23.
226. Kamal S, Kaliki S, Mishra DK, Batra J, Naik MN. Ocular Surface Squamous Neoplasia in 200 Patients A Case-Control Study of Immunosuppression Resulting from Human Immunodeficiency Virus versus Immunocompetency. *ophtha*. 2015;122:1688-1694. doi:10.1016/j.ophtha.2015.04.027
227. Arora T, Sharma S, Sharma N, Titiyal JS. Bilateral recurrent ocular surface squamous cell cancer associated with epidermodysplasia verruciformis. *BMJ Case Rep* 2015. 2015:2014-2016. doi:10.1136/bcr-2014-207495
228. Kasuistiken, Sekundo AG · SP · IS · W. Bilaterale konjunktivale Plattenepithelneoplasie bei Neurodermitis Zwei Fälle mit unterschiedlichem Verlauf. *Ophthalmologe*. 2008;105:1142-1145. doi:10.1007/s00347-008-1748-5



229. El Hamichi S, Messaoudi R, Belmalih M, Iferkhass S, Zerrouk R, Reda K OA. Bilateral enucleation avoided by excision with mitomycin C for bilateral infiltrating conjunctival squamous cell carcinoma in a girl with xeroderma pigmentosum. *J Fr Ophthalmol.* 38(7):135.
230. Lee GA, Williams G, Hirst LW GA. Risk factors in the development of ocular surface epithelial dysplasia. *Ophthalmology.* 101(2):360-364.
231. R Newton, J Ferlay, G Reeves, V Beral DP-. Incidence of squamous cell carcinoma of the eye increases with increasing levels of ambient solar ultraviolet radiation. *Lancet.* 1996:1450-1451.
232. AA. Pizzarello LD JF. Bowen's disease of the conjunctiva: a misnomer. *Jakobiec, FA (Ed), Ocul Adnexal Tumors, Aesculapius Publ Co, Birmingham, Al.* 1978:553-571.
233. Newton R. A review of the aetiology of squamous cell carcinoma of the conjunctiva. *Br J Cancer.* 1996;74(10):1511-1513.
234. Templeton A. Tumours of the Eye and Adnexa. In: *Tumours in a Tropical Country.* ; 1973:203-214.
235. Paisuntornsug T, Karnsawai S. Risk factors for conjunctival squamous cell neoplasia: a matched case-control study. *Br J Ophthalmol.* 2003;87:396-398.
236. Jr JAM, Abdelsalam A, Mclean IW. Adenoid squamous carcinoma of the conjunctiva — a clinicopathological study of 14 cases. *Br J Ophthalmol.* 1997;81(11):1001-1005.
237. Karcioglu ZA, Issa TM. Human papilloma virus in neoplastic and non-neoplastic conditions of the external eye Human papilloma virus in neoplastic and non-neoplastic conditions of the external eye. *Br J Ophthalmol.* 1997;81:595-598.
238. Tumors L, Dushku N, Hatcher SLS, Albert DM, Reid TW. p53 Expression and Relation to Human Papillomavirus Infection in Pingueculae, Pterygia, and Limbal Tumors. *Arch Ophthalmol.* 1999;117:1593-1599.
239. Manderwad GP, Kannabiran C, Honavar SG, Vemuganti GK, Ocular C. Lack of Association of High-Risk

- Human Papillomavirus in Ocular Surface Squamous Neoplasia in India. *Arch Pathol Lab Med.* 2009;133:1246-1250.
240. Moubayed P, Mwakyoma H, Schneider DT. Infections in Precancerous Lesions and Squamous Cell Carcinoma of the Conjunctiva in Subtropical Tanzania. *Am J Clin Pathol.* 2004;122:938-943. doi:10.1309/T189UWWVB71M9VRC
241. Villa LL, Franco EL LI for CRHPSG. Epidemiologic correlates of cervical neoplasia and risk of human papillomavirus infection in asymptomatic women in Brazil. *JNCI J Natl Cancer Inst.* 81(5):332-340.
242. Brandsma JL, Lewis AJ, Abramson A MM. Detection and typing of papillomavirus DNA in formaldehyde-fixed paraffin-embedded tissue. *Arch Otolaryngol Neck Surg.* 116(7):844-848.
243. Malignancy C. Genital Warts and Cervical Cancer 1. 1982.
244. Arends MJ, Wyllie AH BC. Papillomaviruses and human cancer. *Hum Pathol.* 21(7):686-698.
245. Jr JSL, Khan RA, Masand RP, et al. Recognition of nonkeratinizing morphology in oropharyngeal squamous cell carcinoma – a prospective cohort and interobserver variability study. *Histopathology.* 2012;60(3):427-436. doi:10.1111/j.1365-2559.2011.04092.x
246. Jr JSL. Morphologic diversity in human papillomavirus-related oropharyngeal squamous cell carcinoma : Catch Me If You Can ! *Nat Publ Gr.* 2017;30(s1):S44-S53. doi:10.1038/modpathol.2016.152
247. Goodman H. In situ hybridization methods for the detection of somatostatin mRNA in tissue sections using antisense RNA probes. *Histochem J.* 1986;18:597-604.
248. Bobrow MN, Harris TD, Shaughnessy KJ LG. Catalyzed reporter deposition, a novel method of signal amplification application to immunoassays. *J Immunol Methods.* 125(1-2):279-285.
249. P J Poddighe, J Bulten, H MJ Kerstens, J C M Robben W, Melchers G, J AG, Hanselaar M, Department. Human papilloma virus detection by in situ hybridisation signal amplification based on biotinylated tyramine deposition. *J Clin Pathol Mol Pathol.* 1996;49:M340-M344.

250. Aoyama C, Liu P, Ostrzega N, Holschneider CH. Histologic and Immunohistochemical Characteristics of Neoplastic and Nonneoplastic Subgroups of Atypical Squamous Lesions of the Uterine Cervix. *Am J Clin Pathol.* 2005;123:699-706. doi:10.1309/FB900YXTF7D2HE99
251. Guimarães MCM, Gonçalves MAG, Soares CP, Bettini JSR, Duarte RA, Soares EG. Immunohistochemical Expression of p16 INK4a and bcl-2 According to HPV Type and to the Progression of Cervical Squamous Intraepithelial Lesions. *J Histochem Cytochem.* 2005;53(4):509-516. doi:10.1369/jhc.4A6312.2005
252. Jr JSL, Chernock RD, Ma X, Flanagan JJ, Luo Y. Partial p16 staining in oropharyngeal squamous cell carcinoma: extent and pattern correlate with human papillomavirus RNA status. *Mod Pathol.* 2012;25(9):1212-1220. doi:10.1038/modpathol.2012.79
253. Schache AG, Liloglou T, Risk JM, et al. Evaluation of Human Papilloma Virus Diagnostic Testing in Oropharyngeal Squamous Cell Carcinoma: Sensitivity, Specificity, and Prognostic Discrimination. *Clin Cancer Res.* 2011;17(19):6262-6271. doi:10.1158/1078-0432.CCR-11-0388
254. K-T Kuo, H-C Chang, C-H Hsiao M-CL. Increased Ki-67 proliferative index and absence of P16INK4 in CIN-HPV related pathogenic pathways different from cervical squamous intraepithelial lesion. *Br J Ophthalmol.* 2006;90:894-899. doi:10.1136/bjo.2005.086314
255. Chu SJHLP, Shiu HWT, Lang S. Expression of cell cycle-regulatory proteins, MIB-1, p16, p53, and p63, in squamous cell carcinoma of conjunctiva: not associated with human papillomavirus infection. *Virchows Arch.* 2006;448:301-305. doi:10.1007/s00428-005-0104-2
256. Shields CL, Ramasubramanian A, Mellen PL, Shields JA. Arising in Immunosuppressed Patients (Organ Transplant, Human Immunodeficiency Virus Infection). *OPHTHA.* 2011;118(11):2133-2137.e1. doi:10.1016/j.ophtha.2011.04.001
257. Choi CJ, Jakobiec FA, Zakka FR, Foster CS, Chodosh J, Freitag SK. Conjunctival Squamous Cell Neoplasia Associated With Ocular Cicatricial Pemphigoid. *Ophthalm Plast Reconstr Surg.* 2017;33(6):157-160. doi:10.1097/IOP.0000000000000926

258. Parkin DM, Wabinga H, Nambooze S, Wabwire-mangen F. AIDS-related cancers in Africa : maturation of the epidemic in Uganda. *AIDS*. 1999;13:2563-2570.
259. Poole TRG. Conjunctival squamous cell carcinoma in Tanzania. *Br J Ophthalmol*. 1999;83:177-179.
260. HR Wabinga, DM Parkin FW-M and SN. Trends in cancer incidence in Kyadondo County,. *Br J Cancer*. 2000;82(9):1585-1592.
261. Pola EC, Masanganise R RS. The trend of ocular surface squamous neoplasia among ocular surface tumour biopsies submitted for histology from Sekuru Kaguvi Eye Unit, Harare between 1996 and 2000. *Cent Afr J Med*. 2003;49(1-2):1-4.
262. K K. Treatment of conjunctival intraepithelial neoplasia in Africa. *Br J Ophthalmol (electronic Lett)*. 22:120-121.
263. Newton R, Grulich A, Beral V, Sindikubwabo B, Ngilimana PJ, Nganyira A PD. Cancer and HIV infection in Rwanda. *Lancet*. 345(8961):1378-1379.
264. Piot P, Kapita BM, Ngugi EN, Mann JM, Colebunders R WR. *AIDS in Africa: A Manual for Physicians*. World Health Organization; 1992.
265. Guech-ongey M, Engels EA, Goedert JJ, Biggar RJ, Mbulaiteye SM. Elevated risk for squamous cell carcinoma of the conjunctiva among adults with AIDS in the United States. *Int J Cancer*. 2008;122:2590-2593. doi:10.1002/ijc.23384
266. Winward KE CV. Conjunctival squamous cell carcinoma in a patient with human immunodeficiency virus infection. *Am J Ophthalmol*. 107(5):554-555.
267. Goedert J CT. Conjunctival malignant disease with AIDS in USA. *Lancet*. 346(8969):257-258.
268. Ateenyi-Agaba. Conjunctival squamous-cell carcinoma associated with HIV infection in Kampala, Uganda. *Lancet*. 1995;345:695-696.

269. Waddell KM, Lewallen S, Lucas SB, Herrington CS, Liomba G. Carcinoma of the conjunctiva and HIV infection in Uganda and Malawi. *Br J Ophthalmol*. 1996;80:503-508.
270. Krishnan S, Jeffrey TS, Denise LJ, et al. Incidence of Non-AIDS-Defining Cancer in Antiretroviral Treatment-Naïve Subjects after Antiretroviral Treatment Initiation : An ACTG Longitudinal Linked Randomized Trials Analysis. *Oncology*. 2011;80(1-2):42-49. doi:10.1159/000328032
271. Powles T, Robinson D, Stebbing J, Shamash J, Nelson M, Gazzard B. Highly Active Antiretroviral Therapy and the Incidence of Non – AIDS-Defining Cancers in People With HIV Infection. *J Clin Oncol*. 2009;27(6):4-8. doi:10.1200/JCO.2008.19.6626
272. Deeken JF, Tjen-a-looi A, Rudek MA, et al. The Rising Challenge of Non – AIDS-De fi ning Cancers in HIV-Infected Patients. *CID*. 2012;55(9):1228-1235. doi:10.1093/cid/cis613
273. Engels EA, Biggar RJ, Hall HI, et al. Cancer risk in people infected with human immunodeficiency virus in the United States. *Int J Cancer*. 2008;123:187-194. doi:10.1002/ijc.23487
274. Sun J, Burke MS, Zhu H, Loree TR, Brandwein MS. Human Pathology : Case Reports Conjunctival squamous carcinoma in an HIV + woman : Association with high-risk human papillomavirus. *Hum Pathol Case Reports*. 2017;7:64-68. doi:10.1016/j.ehpc.2016.07.005
275. Ateenyi-Agaba C, Weiderpass E, Smet A, et al. Epidermodysplasia verruciformis human papillomavirus types and carcinoma of the conjunctiva : a pilot study. *Br J Cancer*. 2004;90:1777-1779. doi:10.1038/sj.bjc.6601743
276. Tornesello ML, Duraturo ML, Waddell KM, et al. Evaluating the role of human papillomaviruses in conjunctival neoplasia. *Br J Cancer*. 2006;94:446-449. doi:10.1038/sj.bjc.6602921
277. Franceschi S, Arslan A, Othieno E, et al. Human papillomavirus infection and squamous cell carcinoma of the conjunctiva. *Br J Cancer*. 2009;102(2):262-267. doi:10.1038/sj.bjc.6605466
278. International Agency for Research on Cancer IWG on the E of CR to H. IARC monographs on the evaluation of carcinogenic risks to humans. *World Heal Organ*. 2007.

279. Inke Diana Schaper, Marcuzzi GP, Weissenborn J, et al. Development of Skin Tumors in Mice Transgenic for Early Genes of Human Papillomavirus Type 8. *Cancer Res.* 2005;65(4):1394-1401.
280. Malisius R, Merz H, Heinz B, Gafumbegete E, Koch BU, Feller AC. Constant Detection of CD2, CD3, CD4, and CD5 in Fixed and Paraffin-embedded Tissue Using the Peroxidase-mediated Deposition of Biotin – Tyramide. *J Histochem Cytochem.* 1997;45(12):1665-1672.
281. Kasprzak A, Zabel M, Wysocki J, Olejnik A, Gorczy J, Zabel M. Detection of DNA, mRNA and early antigen of the human cytomegalovirus using the immunomax technique in autopsy material of children with intrauterine infection. *Virchows Arch.* 2000;437:482-490. doi:10.1007/s004280000265
282. Wang X, Ouyang H, Yamamoto Y, et al. Residual Embryonic Cells as Precursors of a Barrett ' s-like Metaplasia. *Cell.* 2011;145(7):1023-1035. doi:10.1016/j.cell.2011.05.026
283. Herfs M, Yamamoto Y, Laury A, Wang X, Nucci MR, McLaughlin-drubin ME. A discrete population of squamocolumnar junction cells implicated in the pathogenesis of cervical cancer. *PNAS.* 2012;109(26):10516-10521. doi:10.1073/pnas.1202684109
284. Mirkovic J, Howitt BE, Roncarati P, et al. Carcinogenic HPV infection in the cervical squamo-columnar junction. *J Pathol.* 2015;236:265-271. doi:10.1002/path.4533
285. Taylor S, Wang C, Wright TC, Denny L, Tsai W, Kuhn L. Reduced acquisition and reactivation of human papillomavirus infections among older women treated with cryotherapy : results from a randomized trial in South Africa. *BMC Med.* 2010;8(40):2-10.
286. Davanger M EA. Role of the pericorneal papillary structure in renewal of corneal epithelium. *Nature.* 1971;229:560-561.
287. Cotsarelis G, Cheng S-Z, Dong G, Sun T-T, Lavker RM. Existence of Slow-Cycling Limbal Epithelial Basal Cells That Can Be Preferentially Stimulated to Proliferate : Implications on Epithelial Stem Cells. *Cell.* 1989;57:201-209.

288. Li W, Hayashida Y, Chen Y, Tseng SCG. Niche regulation of corneal epithelial stem cells at the limbus. *Cell Res.* 2007;17:26-36. doi:10.1038/sj.cr.7310137
289. Dushku N, John MK, Schultz GS, Reid TW. Pterygia Pathogenesis: Corneal Invasion by Matrix Metalloproteinase Expressing Altered Limbal Epithelial Basal Cells. *Arch Ophthalmol.* 2001;119:695-706.
290. Chaturvedi AK, Ph D. Beyond Cervical Cancer : Burden of Other HPV-Related Cancers Among Men and Women. *J Adolesc Heal.* 2010;46(4):S20-S26. doi:10.1016/j.jadohealth.2010.01.016
291. Madreperla SA, Green WR DR& SK. Human papillomavirus in primary epithelial tumours of the lacrimal sac. *Ophthalmology.* 1993;100:569-573.
292. Brehm A, Nielsen SJ, Miska EA, et al. The E7 oncoprotein associates with Mi2 and histone deacetylase activity to promote cell growth. *EMBO J.* 1999;18(9):2449-2458.
293. Gillison ML, Trotti AM, Harris J, Eisbruch A, Harari PM, Adelstein DJ, Sturgis EM, Burtness B, Ridge JA, Ringash J GJ. Radiotherapy plus cetuximab or cisplatin in human papillomavirus-positive oropharyngeal cancer (NRG Oncology RTOG 1016): a randomised, multicentre, non-inferiority trial. *The Lancet.* 2018.
294. Shields JA, Demirci H, Marr BP, Eagle RC, Shields CL. Sebaceous Carcinoma of the Ocular Region : A Review. *Surv Ophthalmol.* 2005;50(2):103-122. doi:10.1016/j.survophthal.2004.12.008
295. Abe M, Ohnishi Y, Hara Y, Shinoda Y JK. Malignant tumor of the eyelid--clinical survey during 22-year period. *Jpn J Ophthalmol.* 1983;27(1):175-184.
296. Ni C, Searl SS, Kuo PK, Chu FR, Chong CS AD. Sebaceous cell carcinomas of the ocular adnexa. *Int Ophthalmol Clin.* 1982;22(1):23-61.
297. Rao NA, Hidayat AA, McLean IW Z LE. Sebaceous carcinomas of the ocular adnexa: A clinicopathologic study of 104 cases, with five-year follow-up data. *Hum Pathol.* 1982;13(2):113-122.
298. Jakobiec FA, Mendoza PR. Eyelid sebaceous carcinoma: Clinicopathologic and multiparametric immunohistochemical analysis that includes adipophilin. *Am J Ophthalmol.* 2014;157(1):186-208.e2.

doi:10.1016/j.ajo.2013.08.015

299. Kiyosaki K, Nakada C, Hijiya N, et al. Analysis of p53 mutations and the expression of p53 and p21WAF1/CIP1 protein in 15 cases of sebaceous carcinoma of the eyelid. *Investig Ophthalmol Vis Sci.* 2010;51(1):7-11. doi:10.1167/iovs.09-4127
300. Jung M, Sik H, Sook E, et al. Pathology – Research and Practice Comparison of HER2 gene amplification and KRAS alteration in eyelid sebaceous carcinomas with that in other eyelid tumors. *Pathol -- Res Pract.* 2015;211(5):349-355. doi:10.1016/j.prp.2014.10.006
301. Liao J, Liao S, Hsiao C. Hypermethylation of the CDKN2A gene promoter is a frequent epigenetic change in periocular sebaceous carcinoma and is associated with younger patient age. *Hum Pathol.* 2014;45(3):533-539. doi:10.1016/j.humpath.2013.10.019
302. Jung M, Sook E, Jin S, et al. Pathology – Research and Practice Mutation analysis of CTNNB1 gene and the ras pathway genes KRAS, NRAS, BRAF, and PIK3CA in eyelid sebaceous carcinomas. *Pathol -- Res Pract.* 2017;213(6):654-658. doi:10.1016/j.prp.2017.02.015
303. Gonzalez-Fernandez F, Kaltreider SA, Patnaik BD et al. Sebaceous carcinoma. Tumor progression through mutational inactivation of p53. *Ophthalmology.* 1998;105(3):497–506.
304. Mckelvie PA, Daniell M, McNab A, Loughnan M, Mckelvie P. Squamous cell carcinoma of the conjunctiva: a series of 26 cases. *Br J Ophthalmol.* 2002;86:168-173.
305. Papilloma S, Jakobiec FA, Mendoza PIAR, Colby KA. Clinicopathologic and Immunohistochemical Studies of Conjunctival Large Cell Acanthoma, Epidermoid Dysplasia, and Squamous Papilloma. *Am J Ophthalmol.* 2013;156(4):830-846.e1. doi:10.1016/j.ajo.2013.05.005
306. Valenzuela, Alejandra A. M.D.; McNab, Alan F.R.A.N.Z.C.O.; Sullivan TJFRANZC. Multidisciplinary Management of Lacrimal Sac/Nasolacrimal Duct Carcinomas. *Ophthalmic Plast Reconstr Surg.* 2014;13(6):454-457.

307. Ryan SJ FR. Primary epithelial neoplasms of the lacrimal sac. *Am J Ophthalmol.* 1973;76:73-88.
308. Flanagan JC SD. Lacrimal sac tumors. *Ophthalmolo.* 1978;85:1282-1287.
309. Ni C, D'Amico DJ et al. Tumors of the lacrimal sac: a clinicopathological analysis of 82 cases. *Int Ophthalmol Clin.* 1982;22:121-140.
310. Kerr DA, Arora KS, Mahadevan KK, et al. Performance of a Branch Chain RNA In Situ Hybridization Assay for the Detection of High-risk Human Papillomavirus in Head and Neck Squamous Cell Carcinoma. *Am J Surg Pathol.* 2015;39(12):1643-1652.
311. Shields PW, Jakobiec FA, Stagner AM, Yoon MK, Seregard S. ScienceDirect Spitz nevus arising in the eyelid of a teenager. *Surv Ophthalmol.* 2016;61(2):228-235. doi:10.1016/j.survophthal.2015.10.002
312. Lu DW, El-mofty SK, Wang HL. Expression of p16, Rb, and p53 Proteins in Squamous Cell Carcinomas of the Anorectal Region Harboring Human Papillomavirus DNA. *Mod Pathol.* 2003;16(7):692-699. doi:10.1097/01.MP.0000077417.08371.CE
313. Li Y, Nichols MA, Shay JW, Xiong Y. Transcriptional repression of the D-type cyclin-dependent kinase inhibitor p16 by the retinoblastoma susceptibility gene product pRb. *Cancer Res.* 1994;54(23):6078-6082.
314. Willman JH, Heinz D, Golitz LE, Shroyer KR. Correlation of p16 and pRb expression with HPV detection in Bowen's disease. *J Cutan Pathol.* 2006;33(9):629-633. doi:10.1111/j.1600-0560.2006.00499.x
315. Jakobiec FA, Werdich X. Androgen Receptor Identification in the Diagnosis of Eyelid Sebaceous Carcinomas. *AJOPHT.* 2014;157(3):687-696.e2. doi:10.1016/j.ajo.2013.12.009
316. Milman T, Schear MJ, Eagle RC. Diagnostic Utility of Adipophilin Immunostain in Periocular Carcinomas. *Ophthalmology.* 2014;121(4):964-971. doi:10.1016/j.ophtha.2013.10.041

APPENDIX A

Material Transfer Agreement

3615/17-783

Check List to be completed before grants/contracts/MOUs/MOAs are forwarded for signature on behalf of the University of the Western Cape.

CONTRACT(S)/AGREEMENT(S): Materials Transfer Agreement between Massachusetts General Hospital (Harvard Medical School) and the University of the Western Cape in respect of stained and unstained slides for analyzing human papilloma virus in orbital tumours (see attached).

1. Is the relevant Head of Department/Division aware of the grant/contract/MOU/MOA?

YES NO

Signature of HOD: [Signature] Date: 17.5.2017
A. De la Riva
PRINT NAME & POSITION

2. Has the relevant Executive Manager endorsed the grant/contract/MOU/MOA? (e.g the Deputy Vice Chancellor (Academic) for the Faculties; the Director: International Office for MOUs with other Universities)

YES NO

Signature of Executive Manager: [Signature] Date: 23/5/17
M. van der Merwe
PRINT NAME & POSITION

Has the Director: Legal Services assessed all risks and approved the grant/contract/MOU/MOA for signature?

YES NO

Signature of Director: Legal Services: [Signature] Date: 19/5/2017
Mr S Rajie
Director: Legal Services

Name of Member submitting the grant/contract/MOU/MOA for signature:

Signature of Member: [Signature] Date: 17 May 2017
Print name and surname: Donavan Hiss

Extention/Telephone #: 2334 Email address: dhiss@uwc.ac.za



Department of Pathology
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William C. Faquin, M.D., Ph.D.
Director, Head and Neck Pathology
Massachusetts Eye and Ear
Massachusetts General Hospital
Professor of Pathology
Harvard Medical School

May 15, 2017

Dr. Donovan Hiss
Dept of Medical Biosciences
University of the Western Cape, Room 4016
Life Sciences Building
Modderdam Road
Bellville, 7535, South Africa



Dear Dr. Hiss:

You have requested that MGH ("Institution") provide you with the following tissue samples **consisting of H&E stained slides and unstained slides of formalin-fixed tissue** in connection with the following not-for-profit project (*i.e.*, research or teaching) at your academic institution: **investigation of the role of HPV in orbital tumors being performed in collaboration between MGH and the University of the Western Cape ("Project")**. Because of the nature of your intended use at your institution, we can provide these samples if you agree to a few terms below. Please review and sign this letter below and return it to me, and I will promptly send the tissues you requested. If you later request additional samples of the same type in connection with the Project specified above, this letter also will apply to such requests.

1. You agree that you will use these samples only for the Project described above. You understand that **THESE SAMPLES ARE NOT INTENDED FOR USE IN, AND YOU AGREE THAT YOU WILL NOT USE THEM IN, HUMAN SUBJECTS**. You also agree that the samples will be used only by you and by individuals under your direct supervision on your Project team, and you agree not to transfer these samples to any other person, institution, or entity. You agree that you, and individuals under your direct supervision on your Project team, will use the samples in compliance with all applicable laws and regulations.
2. a) If you are requesting only coded information with the samples – that is, information linked by a code to identifiable information at our institution – then you agree that you, and your institution, will not have or seek access to any identifiable information (such as the key to the code) under any circumstances. You agree to never use the samples or any material derived from the samples (e.g. DNA, RNA or the information provided with the samples) to attempt to ascertain the identity of the individual from whom they were obtained.

1 of 4 D.H.

- b) If you are requesting select information that falls within a "limited data set" – as explained in the attachment – then you agree to sign the attachment, which is a "Data Use Agreement" required under the HIPAA Privacy Rule.
3. You agree to review the details of the Project with your own institution and your institution's IRB to determine whether they will require further institutional and/or IRB review. You also agree to acknowledge the source of the samples in any publications reporting use of them.
 4. The samples are provided at no cost or with an optional transmittal fee solely to reimburse preparation and distribution costs. If a fee is requested, the amount will be indicated here: N/A.
 5. You accept these samples as-is. You understand that these samples may have hazardous properties and may carry transmissible infectious agents. I AND INSTITUTION MAKE NO REPRESENTATIONS AND EXTEND NO WARRANTIES OF ANY KIND, EITHER EXPRESSED OR IMPLIED, INCLUDING NO EXPRESS OR IMPLIED WARRANTIES OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE, OR THAT THE USE OF THE MATERIAL WILL NOT INFRINGE ANY PATENT, COPYRIGHT, TRADEMARK, OR OTHER PROPRIETARY RIGHTS. INSTITUTION, ITS PERSONNEL, AND I DISCLAIM ALL LIABILITY FOR ANY CLAIMS OF DAMAGES THAT MAY ARISE FROM YOU, YOUR INSTITUTION, OR THIRD PARTIES RELATING TO THE USE, STORAGE, OR DISPOSAL OF THE SAMPLES.

As stated above, once you sign and return this letter, along with the completed Data Use Agreement attached (if applicable), I will forward the samples you requested.

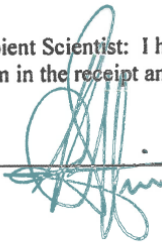
Sincerely,


 William C. Faquin, MD, PhD


 UNIVERSITY of the
 WESTERN CAPE

Certification of Recipient Scientist: I have read and understood the terms outlined in this letter, and I agree to abide by them in the receipt and use of the samples described above.


Recipient Scientist



Donatien C. Hiss PhD

17 May 2017
 Date

Accepted and endorsed by:


 Professor Jesse Frantz Vivienne Lawack
 Deputy Vice Chancellor
 University of the Western Cape

2 of 4 D.H.

DATA USE AGREEMENT: LIMITED DATA SET

As required by the Privacy Rule issued under the Health Insurance Portability and Accountability Act (HIPAA), as the Recipient Scientist receiving a Limited Data Set, you agree to the following terms of this Data Use Agreement.

I. You have requested the following data ("Limited Data Set") for use with tissue samples in the Project described above, and you represent that such data are the minimum necessary to achieve the stated purpose:

- Dates (e.g., admission date, birth date)
- Geographic information excluding street address (e.g., city, zip code)
- Other information not excluded from a limited data set (the following information about an individual or the individual's relatives, employers, or household members, is not permitted in a limited data set: name, street address, telephone/fax numbers, electronic mail address, Social Security number, medical record number, health plan beneficiary number, account numbers, certificate/license numbers, vehicle identifiers and serial numbers, including license plate number, device identifiers and serial numbers, URLs and IP addresses, biometric identifiers, including finger and voice prints, full face photos and comparable images).

II. Only you, and individuals under your direct supervision on your Project team, will have access to the Limited Data Set.

III. In consideration of Institution sending the Limited Data Set to you, you agree:

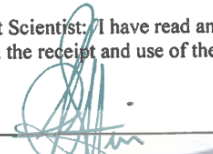
- A. To use and disclose the Limited Data Set only for the purpose specified above, and to not use or further disclose such information in a manner that would violate the Privacy Rule if done by the Institution;
- B. To permit only yourself and the Project team to use or receive the Limited Data Set;
- C. To represent, warrant, and covenant that you (i) will not use or disclose the Limited Data Set other than as permitted by this Agreement or as otherwise required by law; (ii) will use appropriate safeguards to prevent use or disclosure of the information other than as provided for by this Agreement; (iii) will report promptly to Institution any use or disclosure not provided for by this Agreement of which you become aware; (iv) will ensure that any agents (allowed only with prior written approval), including a subcontractor, to whom you provide the Limited Data Set agrees to the same restrictions and conditions that apply to you as the Recipient Scientist with respect to such information; and (v) will not use this information to identify or to contact the individuals.
- D. To obtain institutional review board review and approval of research activities when required by law or regulation;

 3 of 4 D.H.

E. To agree that in the event of a breach or violation of this Agreement, Institution has the right to report the problem to the Secretary of Health and Human Services and to take other appropriate action, including but not limited to terminating this Agreement.

Certification of Recipient Scientist: I have read and understood the terms outlined in this letter, and I agree to abide by them in the receipt and use of the samples described above.

Recipient Scientist


Donovan C. Hiss PhD

17 May 2017
Date

Agreed to by:

Name: Dr. Donovan Hiss

Title: Professor

Institution: University of the Western Cape


Phone number: 00272195992334; Fax: 0027219591563

Email address: dhiss@uwc.ac.za

79698



Accepted and Endorsed by:



Professor José Frantz Vilmerne
Deputy Vice Chancellor
University of the Western Cape

UNIVERSITY of the
WESTERN CAPE

4 of 4 D.H

APPENDIX B

Ethics Clearance Certificate



OFFICE OF THE DIRECTOR: RESEARCH
RESEARCH AND INNOVATION DIVISION

Private Bag X17, Bellville 7535
South Africa
T: +27 21 959 2988/2948
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14 June 2017

Prof D Hiss, Dr W Faquin and Dr AH Afrogheh
Medical Biosciences
Faculty of Natural Science

Ethics Reference Number: BM16/4/12

Project Title: The role of high risk human papillomavirus in periocular cancers.

Approval Period: 03 June 2017 – 03 June 2018

I hereby certify that the Biomedical Science Research Ethics Committee of the University of the Western Cape approved the scientific methodology and ethics of the above mentioned research project.

Any amendments, extension or other modifications to the protocol must be submitted to the Ethics Committee for approval.

Please remember to submit a progress report in good time for annual renewal.

The Committee must be informed of any serious adverse event and/or termination of the study.

A handwritten signature in black ink, appearing to read 'Patricia Josias'.

Ms Patricia Josias
Research Ethics Committee Officer
University of the Western Cape

PROVISIONAL REC NUMBER -130416-050

FROM HOPE TO ACTION THROUGH KNOWLEDGE.