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THE ROLE OF HIGH-RISK HUMAN PAPILLOMAVIRUS IN PERIOCULAR CANCERS

by

AMIR H. AFROGHEH

Student Number: 9904096

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Department of Medical Biosciences

Faculty of Natural Sciences

University of the Western Cape

Supervisor	*Co-Supervisor*
Prof Donavon C. Hiss	**Prof William C. Faquin**

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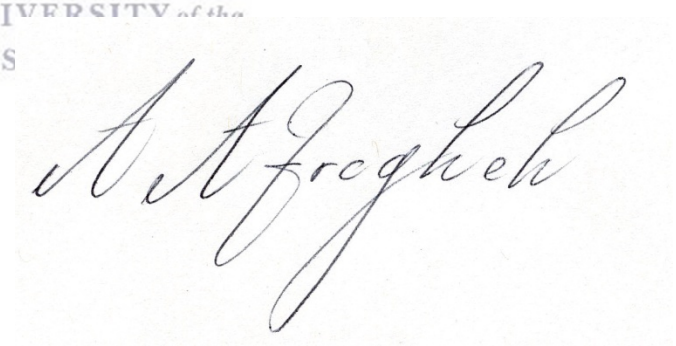
DECLARATION

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.

Amir H. Afrogheh

Student Number: 9904096

: 

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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	Retinoblatoma
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 orophaynx. 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 18cases 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 type16 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 conjuctival- 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 patients[32–35], 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 epithlial 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 oncoprotiens 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 entryin 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 theNucleosome 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 proteosome-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 proteosome-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 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 India[118], 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 bur 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]

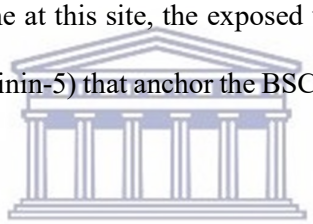
The survival benefit of patients with HPV-positive OPSCC, inspired O'Sullivan et al. to propose a new TNM staging system for HPV-positive OPSCC, resembling the N system for nasopharyngeal carcinoma.[151] O'Sullivan et al. TNM staging system is currently included in the eighth edition of American Joint 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 primaryOPSCC.[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 mRNAindicates 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 arrest[183]. 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. Althoughp16 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 OPSSC. [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-oropharyngeal 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-oropharyngeal 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 circumvallete 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 pyramidine 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 abonormal 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).

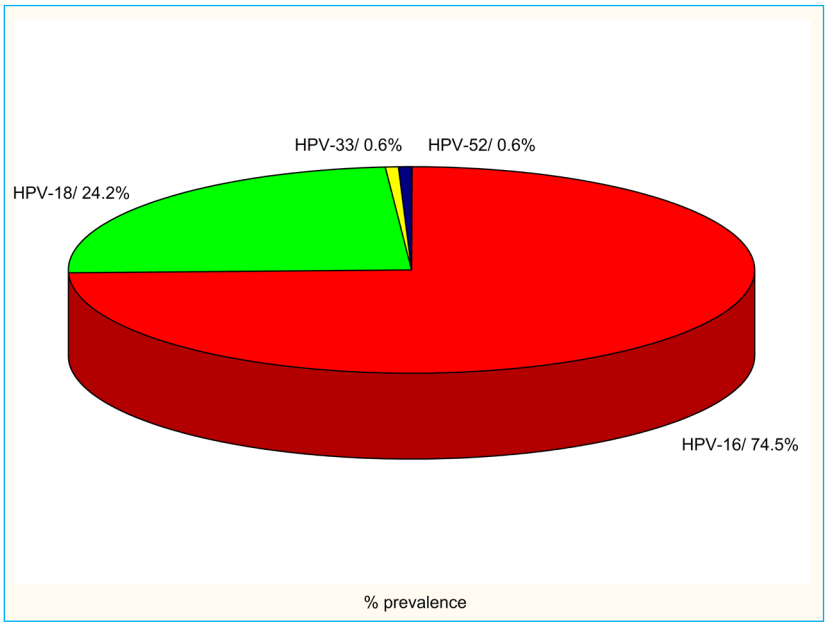


Figure 1.1: Prevalence of HR-HPV types in OSSN

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-keratizing/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 ampilfiable 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 (%)
McDonnel l 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 methods 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 transcritionally 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
Avergae %				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 to10-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 antiretrovial 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-SCC 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 aetiologic role for these viruses in the development of OSSN in young HIV-positive patients (mean age of 33 years).[275]

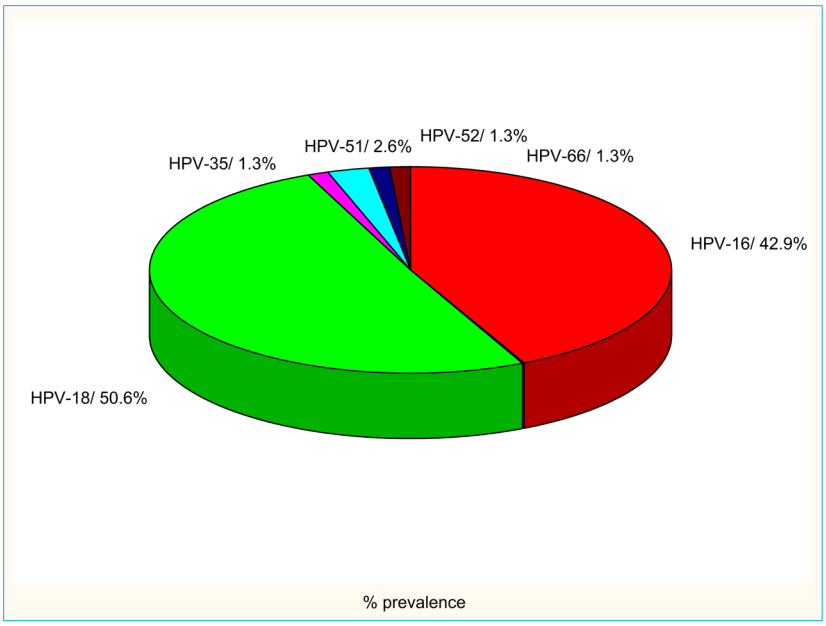


Figure 1.2: Prevalence of HR-HPV types in HIV-associated OSSN

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-keratinzing/undifferentiated cervical- and oropharyngeal SCC. In addition, one of the two non-keratnizing SCC in the study was completely negative for HR-HPV DNA.[240]

Thus, it can 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]

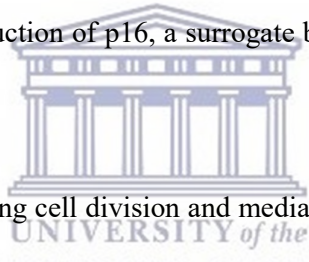
The virus infects the self-renewing BSC through a micro-abrasion/fissure in the epithelium that exposes the epithelial basement membrane [71]. The strategy of infecting self-renewing cells through an open wound, not only ensures long-term viral persistence in actively dividing BSCs but also promotes cellular proliferation as part of the highly organized wound healing process, which could help in the establishment of the viral infection[44]. 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 oncoprotiens 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 proteosome-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 proteosome-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 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.

1.2.13 HR-HPV in Carcinomas of the Lacrimal Sac

In the head and neck, a causal role for HR-HPV has been established in oropharyngeal carcinomas[113]. 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 oncoprotiens, 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] HER2amplification,[300] and epigenetic changes such as hypermethylation of the CDKN2A promoter[301] 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, *CDKN2A*, mismatch repair proteins	1/24 HPV positive (4.2%)
Kwon et al.	14 eyelid SC	PCR	p16, *KRAS, HER2*	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%)

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 (NYEEI).The H & E stained sections were reviewed to confirm the histologic diagnosis and assess the histomorphologic features of each tumour. Unstained 5-µm 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 ≥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-µm 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)

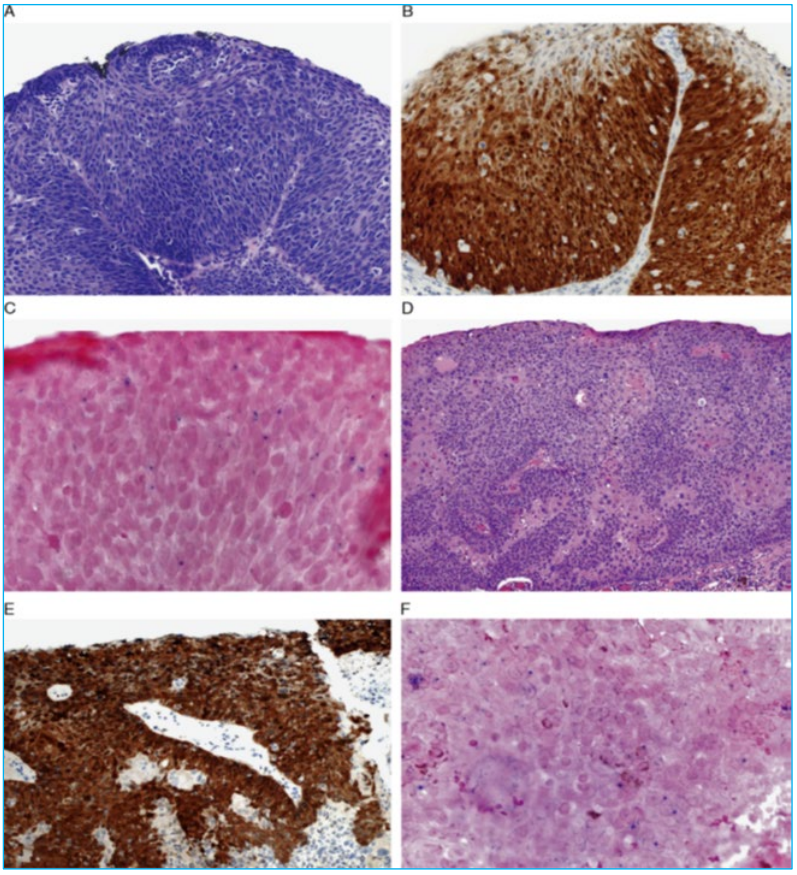
Our cohort of conjunctival ocular surface squamous neoplasms (OSSN) consisted of 25 SCCIS and 18 I-SCC comprising 33 men and 10 women with a mean age of 67 years (range: 39-94 years). Using p16 IHC as a screening test followed by DNA ISH for HR-HPV as a confirmatory test, 30% (n=13/43) of OSSN were determined 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

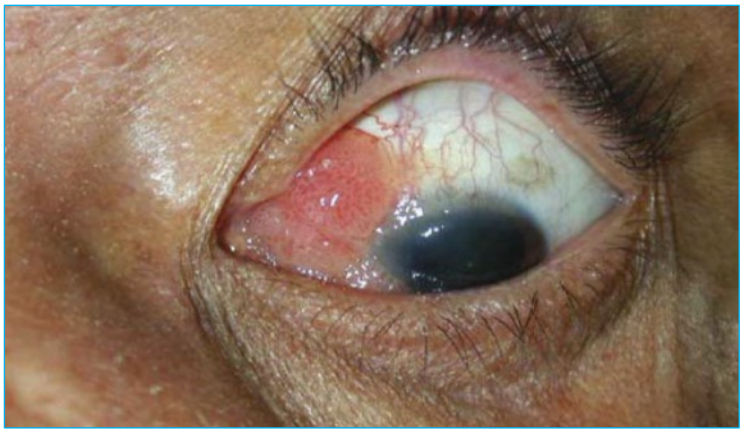
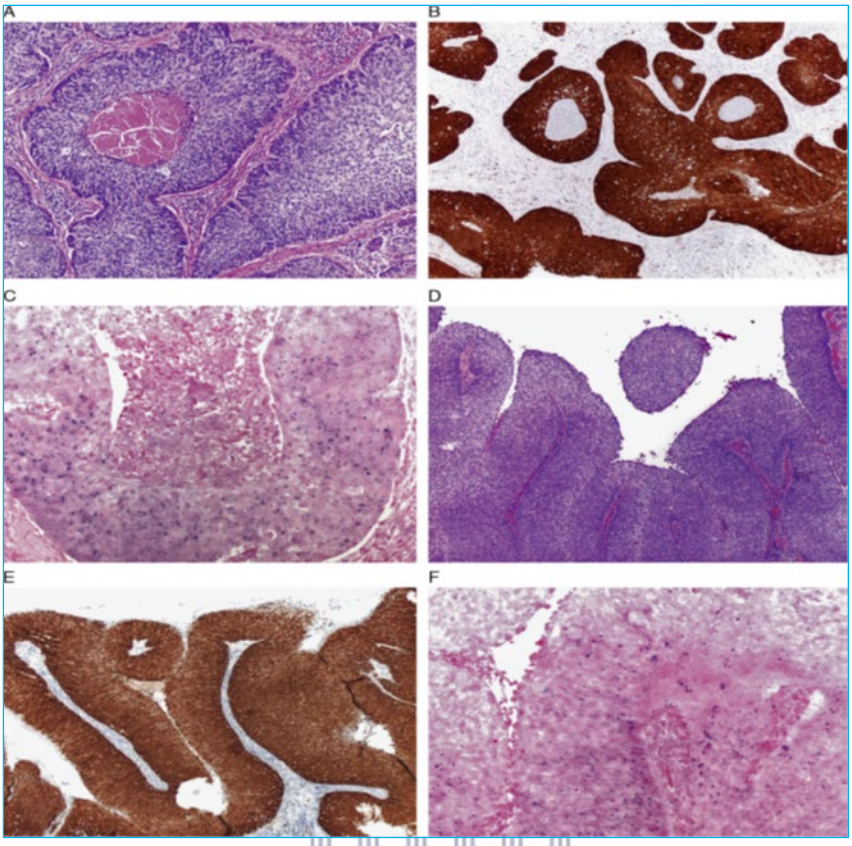


Figure 3.2: Clinical image showing a diffuse pattern of involvement by an HR-HPV-positive SCCIS of the conjunctiva

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.

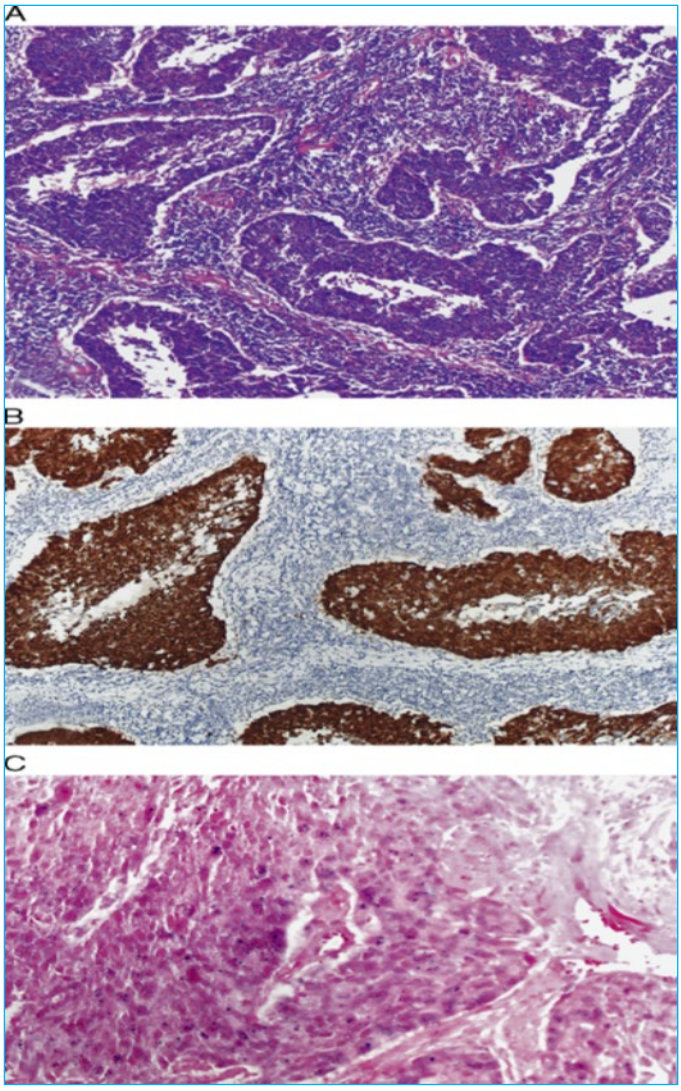
Table 3.2: Clinicopathologic features of 8 cases of HR-HPV-positive lacrimal sac SCC

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

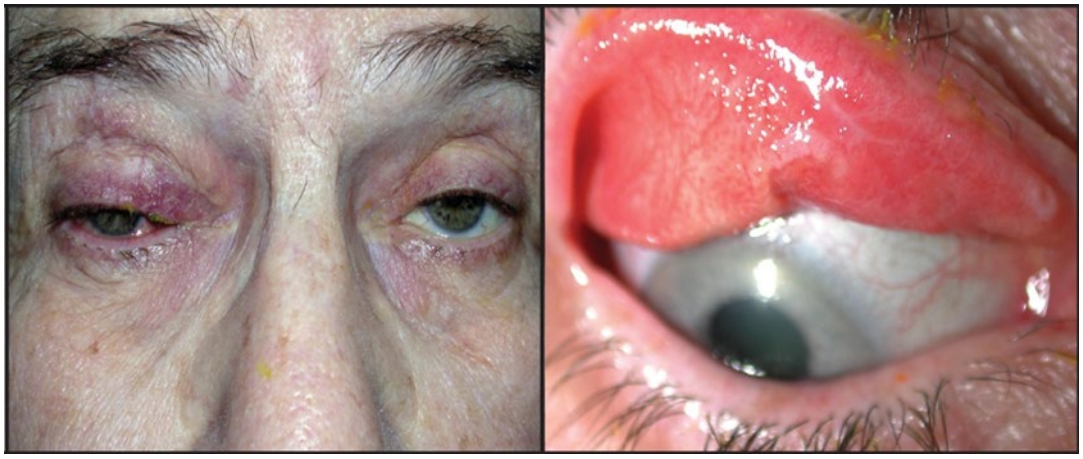
The cohort of periocular SC consisted of 35 cases comprising 12 men and 23 women with a mean age of 78.9 years (range, 46 to 94 y). Tumors more commonly presented in the upper eyelid (Figure 3.5, left and right). In thirteen biopsy specimens, the tumor was confined to the epithelium (Figure 3.6, top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

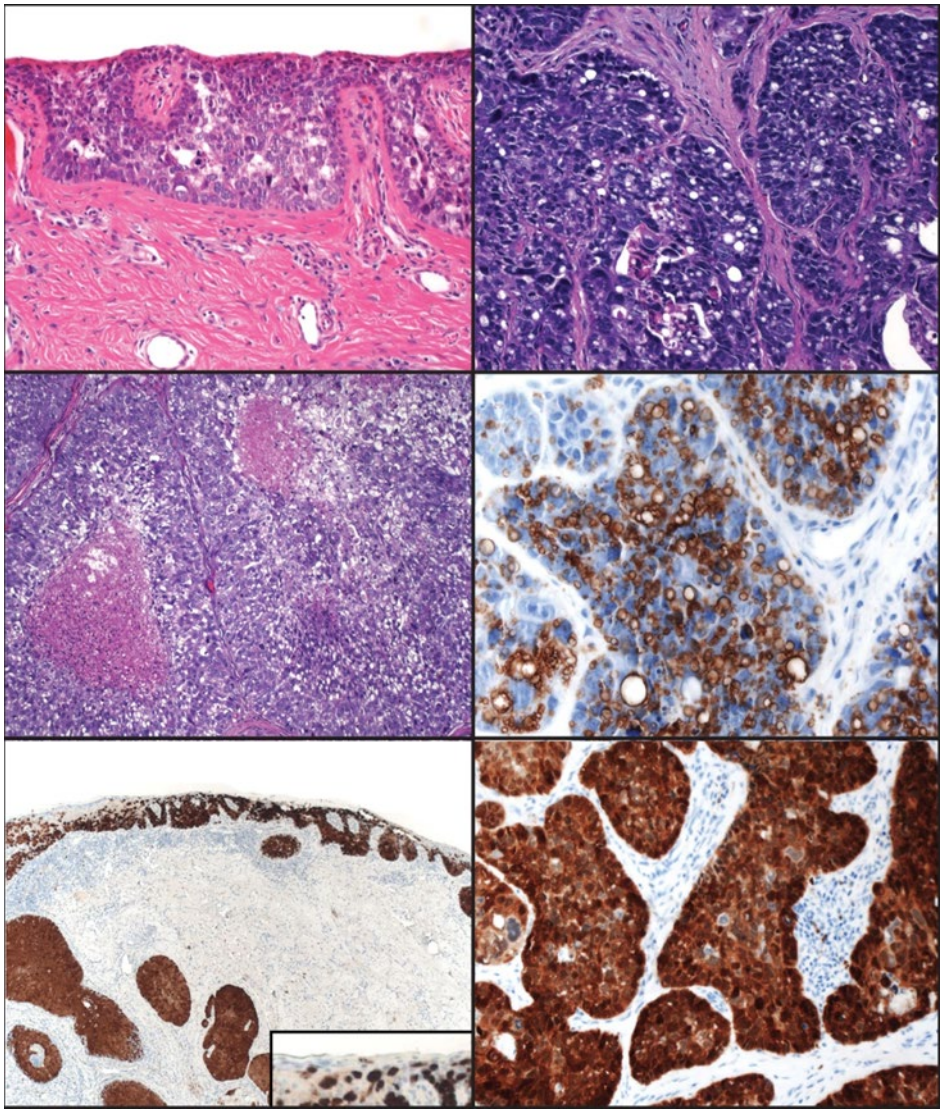


Clinical features of sebaceous carcinoma. (Left) Mild unilateral (right) eyelid margin thickening with erythema mimicking blepharitis; ptosis is also present. (Right) On eversion of the upper eyelid, a diffusely erythematous and irregular superior tarsal conjunctival surface is noted, indicative of intraepithelial (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 at 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 periocular 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.[212]HR-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 study[301] 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 stated[28] 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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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: _____ Date: 17.5.2017

A. Jacobs
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 [X] NO [ ]

Signature of Executive Manager: _____ Date: 23/5/17

Mawa
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: _____ Date: 19/5/2017

Mr S Rajie
Director: Legal Services

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

Signature of Member: _____ Date: 17 May 2017

Print name and surname: Donavon Hiss

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

MASSACHUSETTS GENERAL HOSPITAL

HARVARD MEDICAL SCHOOL

Department of Pathology
55 Fruit Street, WRN-219
Boston, Massachusetts 02114-2696
Tel: 617-573-3957 | Fax: 617-573-3389
Email: wfaquin@partners.org

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. Donavan 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

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

17 May 2017
Date

Donavon C. Hiss PhD

Accepted and endorsed by:

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

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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 — Date: 17 May 2017

Donavon C. Hiss PhD

Agreed to by:
Name: Dr. Donavon 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~~ Vivienne Lawack
Deputy Vice Chancellor
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
F: +27 21 959 3170
E: research-ethics@uwc.ac.za
www.uwc.ac.za

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.

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

PROVISIONAL REC NUMBER -130416-050

FROM HOPE TO ACTION THROUGH KNOWLEDGE.