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THESIS

Human papillomavirus-related neoplasia of the ocular adnexa

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Summary

Human papillomaviruses (HPV) are involved in approximately 5% of solid cancers worldwide. The mucosotropic genotypes infect the stratified epithelium of various locations, where persistent infection may lead to invasive carcinomas. While the causative role of HPV in certain anogenital and head and neck carcinomas is well established, the role of HPV in carcinomas arising in the mucosal membranes of the ocular adnexal tissue (the lacrimal drainage system and the conjunctiva) has been a topic of great uncertainty. Therefore, we conducted a series of studies to assess the correlation between HPV and carcinomas arising in the mucosa of the ocular adnexal tissue and characterize the clinical, histopathological, and genomic features of the tumors in the context of HPV status in a Danish nationwide cohort.

We collected clinical and histopathological data and tumor specimens from patients with carcinomas of the conjunctiva and the lacrimal drainage system, and their potential precursors, identified in Danish nationwide registries. The HPV status of the tumors was determined by the combined use of HPV DNA polymerase chain reaction (PCR), HPV E6/E7 mRNA in-situ hybridization, and p16 immunohistochemistry. The genomic profile was investigated by high-throughput DNA sequencing targeting 523 cancer-relevant genes. The literature to date on carcinomas of the lacrimal drainage system and the conjunctiva was summarized.

In the Danish cohort, 67% of all carcinomas of the lacrimal drainage system and 21% of all conjunctival carcinomas were HPV-positive. HPV16 was the most frequently implicated genotype. A full-thickness expression of the viral oncogenes E6 and E7 was evident in almost all HPV DNA-positive cases. The HPV-positive carcinomas of the conjunctiva and the lacrimal drainage system shared histopathological and genomic features distinct from their HPV-negative counterparts. The HPV-positive carcinomas were characterized by a non-keratinizing morphology, p16 overexpression, high transcriptional activity of HPV E6/E7, and frequent pathogenic variants in the PI3K-AKT signaling cascade. In contrast, the HPV-negative carcinomas were characterized by a keratinizing morphology, lack of p16 and E6/E7 expression, and frequent somatic pathogenic variants in *TP53*, *CDKN2A*, and

Abbreviations: A, Adenine; AIDS, Acquired immunodeficiency syndrome; AJCC, American Joint Committee on Cancer; AR, Androgen receptor; ART, Anti-retroviral therapy; C, Cytosine; CIN, Conjunctival intraepithelial neoplasia; CT, Computed tomography; DNA, Deoxyribonucleic acid; FFPE, Formalin-fixed and paraffin-embedded; G, Guanine; HE, Hematoxylin and eosin; HER2, Human epidermal growth factor receptor 2; HIV, Human immunodeficiency virus; HNSCC, Head and neck squamous cell carcinomas; HPV, Human papillomavirus; HSV, Herpes simplex virus; IARC, International Agency for Research on Cancer; IHC, Immunohistochemistry; IR, Ingvild Ramberg; ISH, In-situ hybridization; LDS, Lacrimal drainage system; MRI, Magnetic resonance imaging; NGS, Next-generation sequencing; OSSN, Ocular surface squamous neoplasia; PCR, Polymerase chain reaction; RB1, Retinoblastoma 1 gene; RNA, Ribonucleic acid; SCC, Squamous cell carcinoma; SH, Steffen Heegaard; SNOMED, Systematized Nomenclature of Medicine; T, Thymine; TP53, Tumor suppressor 53 gene; UNAIDS, Joint United Nations Programme on HIV/AIDS; UVR, Ultraviolet radiation; VLP, Virus-like particles; WHO, World Health Organization.

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RB1. Among the patients with conjunctival tumors, HPV positivity was associated with a younger age at diagnosis and a higher risk of recurrence.

In conclusion, the results support an etiological role of HPV in a subset of conjunctival and LDS carcinomas and their precursor lesions. Our investigations have shown that the HPV-positive carcinomas of the ocular adnexa share genomic and phenotypic characteristics with HPV-positive carcinomas of other anatomical locations. Therefore, these patients may be eligible for inclusion in future basket trials and future treatment regimens tailored to the more frequently occurring HPV-positive carcinomas of other locations. Future research will further elucidate the diagnostic, prognostic, and predictive role of HPV in these carcinomas.

Dansk resumé

Human papillomavirus (HPV) forårsager ca. 5% af alle non-hæmatologiske cancertilfælde på verdensplan. De slimhindeafficerende genotyper inficerer flerlagede pladeepitheler i forskellige anatomiske lokalisationer, og en persisterende infektion kan medføre cancerudvikling. Den kausale rolle for HPV i udviklingen af visse anogenitale og for hoved-hals cancer er veletableret, men rollen i udviklingen af carcinomer i det okulære adnexa (conjunctiva og tårevejene) er stadig behæftet med usikkerhed. Vi udførte derfor en serie af studier for at undersøge sammenhængen mellem HPV og udviklingen af carcinom i conjunctiva og tårevejene og karakterisere den kliniske, histologiske og genetiske profil af tumorerne baseret på HPV-status i en landsdækkende, dansk kohorte.

Ved brug af landsdækkende patientregistre, indsamlede vi kliniske og histopatologiske data samt tumormateriale fra patienter diagnosticeret med carcinom i conjunctiva eller tårevejene og deres potentielle forstadier. Undersøgelser for HPV i tumormaterialet blev foretaget ved p16 immunhistokemi, HPV DNA polymerase chain reaction (PCR) og ved HPV E6/E7 mRNA in-situ hybridisering. Den genetiske profil blev undersøgt ved high-throughput DNA-sekventering målrettet 523 cancer-relevante gener. Litteraturen omhandlende associationen mellem HPV og conjunctival intraepithelial neoplasi og carcinom blev gennemgået.

I det danske materiale var 67% af tårevejscarcinomerne og 21% af alle conjunctivale carcinomer HPV-positive. I begge lokalisationer var HPV16 den hyppigste genotype. Alle HPV-positive tumorer, fraset én, udtrykte ekspression af de virale onkogener E6 og E7. Histopatologiske og genetiske undersøgelser viste at de HPV-positive carcinomer udgået fra conjunctiva og tårevejene delte genotypiske og fænotypiske træk der adskilte dem fra de HPV-negative carcinomer. De HPV-positive carcinomer var karakterisereret af en ikke-keratiniserende morfologi, p16-ekspression, udtalt ekspression af HPV E6/E7 og hyppige patogene varianter i PI3K-AKT signalleringskaskaden. Derimod var de HPV-negative carcinomer karakteriseret af en keratiniserende morfologi og hyppige patogene varianter i *TP53*, *CDKN2A*, og *RB1*.

For at konkludere, støtter vores resultater op om at HPV spiller en kausal rolle i subgrupper af carcinomer og deres forstadier der udgår fra conjunctiva og tårevejene. Vores undersøgelser har vist, at de HPV-positive carcinomer deler genetiske og fænotypiske karakteristika med HPV-positive carcinomer i andre anatomiske lokalisationer. Det er derfor muligt, at disse patienter kan indgå i fremtidige basket-trials og kan drage nytte af de behandlingsmetoder der udvikles til hyppigere forekomne HPV-positive carcinomer. Fremtidig forskning vil videre afgøre den diagnostiske, prognostiske, og prædiktive værdi af HPV i carcinomer i det okulære adnexa.

INTRODUCTION

Historical background

The discovery of the oncogenic potential of human papillomavirus (HPV) over the last 40 years, ultimately leading to the invention of prophylactic vaccines, has led us into a new era of cancer prevention. However, the foundation for these discoveries was laid long before (Figure 1). The existence of *papillomaviruses* goes back more than 40 million years, and the divergence of carcinogenic HPVs from their ancestral host occurred more than half a million years ago – coinciding with the transition from Neanderthals to modern *homo sapiens* (Chen et al., 2018). Both the ancient Greeks and Romans were aware of the contagiousness of genital condylomas (from Greek "kondulōma," meaning knuckle) (Bäfverstedt, 1967), and the appearance and treatment were thoroughly described by Hippocrates (460–388 BC) (Hippocrates, 1868). However, identifying the infectious particle and its association to *cancer*, also in other anatomical locations than the uterine cervix, was more than 2000 years to come.

In 1981, the contagious nature of human warts was experimentally confirmed (Payne, 1891), and some years later documented to be of viral origin (Ciuffo, 1907; Waelsch, 1918). Following these, in hindsight, important observations, little attention was paid to further research in human warts, probably because warts were not considered genuine tumors, and the link between virus and cancer development was not yet established.

It was not until the 1950–1960s that studies of human oncogenic viruses got a new revival due to the discovery of virus-induced malignancies in animals (Olson Jr. & Cook, 1951; Parsons & Kidd, 1943; Rous, 1910) and advances in molecular biology techniques. Epidemiological studies now linked cervical cancer development to an infectious source (Gagnon, 1950; Rotkin, 1967; Towne, 1955). The scientific community was centered around herpes simplex virus 2 (HSV2) as the causative agent for cervical carcinoma development (Nahmias et al., 1974); however, this was not a belief shared by the German virologist Harald zur Hausen, who had failed in several attempts to demonstrate HSV2 in cervical carcinoma samples. Instead, he had noted similarities between genital condyloma and cervical carcinoma, including anecdotal reports of malignant transformation of condyloma (zur Hausen, 1976). His research led to the isolation of HPV6 from genital warts in 1980 (Gissmann & zur Hausen, 1980), and, shortly after, the phylogenetically similar HPV11 was isolated from laryngeal papillomas (Gissmann et al., 1982). Based on these discoveries, HPV16 was identified in 1983 (Dürst et al., 1983) followed by HPV18 in 1984 (Boshart et al., 1984), eventually documenting HPV as the causative agent of cervical cancer development (zur Hausen, 1987). In the 1990s, the first studies of the prophylactic HPV vaccine Gardasil were launched (Zhou et al., 1991), leading to subsequent approval for use in 80 different countries by 2007 (Markowitz et al., 2007). We have now entered a new era in our understanding of viral-induced cancers, which includes cancers and their precursors developing outside of the genital region, such as the ocular adnexa, which is the subject of the present thesis.

Human papillomavirus

Human papillomavirus belongs to the family of *Papillomaviridae* and is classified according to the International Committee on Taxonomy of Viruses (International Committee on Taxonomy of Viruses, 2021) (family, genera, and species) and the International HPV Reference Center (International HPV Reference Center, 2021) (genotype and variant). All HPV can be found among the alpha, beta, gamma, mu, and nu genera, and HPV genotypes with mucosal tropism belong to the alpha genus (Figure 2).

The International Agency for Research on Cancer (IARC) under the World Health Organization (WHO) has classified the mucosotropic alpha-genus HPV genotypes into high-risk (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59) and low-risk (HPV6 and 11, among others) genotypes based on their carcinogenic potential in the uterine cervix (International Agency for Research on Cancer, 2012a). Of the high-risk genotypes, HPV16 and 18 harbor the highest malignant potential, which together are responsible for more than 70% of all cervical carcinomas and 95% of all oropharyngeal SCC (Bonde et al., 2020; Hoffmann & Quabius, 2021).

HPV are circular, double-stranded DNA viruses (Van Doorslaer et al., 2018). The alpha HPV genome consists of 7000–8000 base pairs and includes a long, transcribed control region and eight genes labeled "early" (E1/E2/E4/E5/E6/E7) and "late" (L1/L2) genes based on when the transcripts are expressed in the viral lifecycle. The nucleotide sequence identity of *L1*, *L2*, *E1*, and *E2* determines the phylogenetic placement of the genotypes (Figure 2). The *L1* and *L2* genes encode the capsid protein that forms the icosahedral structure of the HPV particle that self-assembles in productive infections before the viral particle is shed. The "core" *E1–E4* genes encode the proteins responsible for HPV replication and viral release, while the "accessory" *E5–E7* genes encode proteins that alter the extra- and intracellular environment, making it suitable for viral replication (Egawa et al., 2015). Due to the importance of *E6* and *E7* in transforming the keratinocyte, these two oncogenes are described in more detail below.

Papillomaviruses show strict species and tissue tropism: HPV only infects (stratified) epithelia in humans, with rare transmission between species. Transition zones represent locations with increased susceptibility for HPV-related neoplasia, clearly exemplified in the transformation zone between the uterine endo- and ectocervix, where more than 90% of the cervical malignancies develop (Burghardt & Ostör, 1983; Doorbar et al., 2021; Mirkovic et al., 2015). HPV particles are believed to enter the undifferentiated basal epithelial cells after microtraumas to the superficial cell layers. Herein, the particles initiate a low-copy-number and non-productive infectious state and thereby evade responses from the immune system. The replication of the viral DNA follows

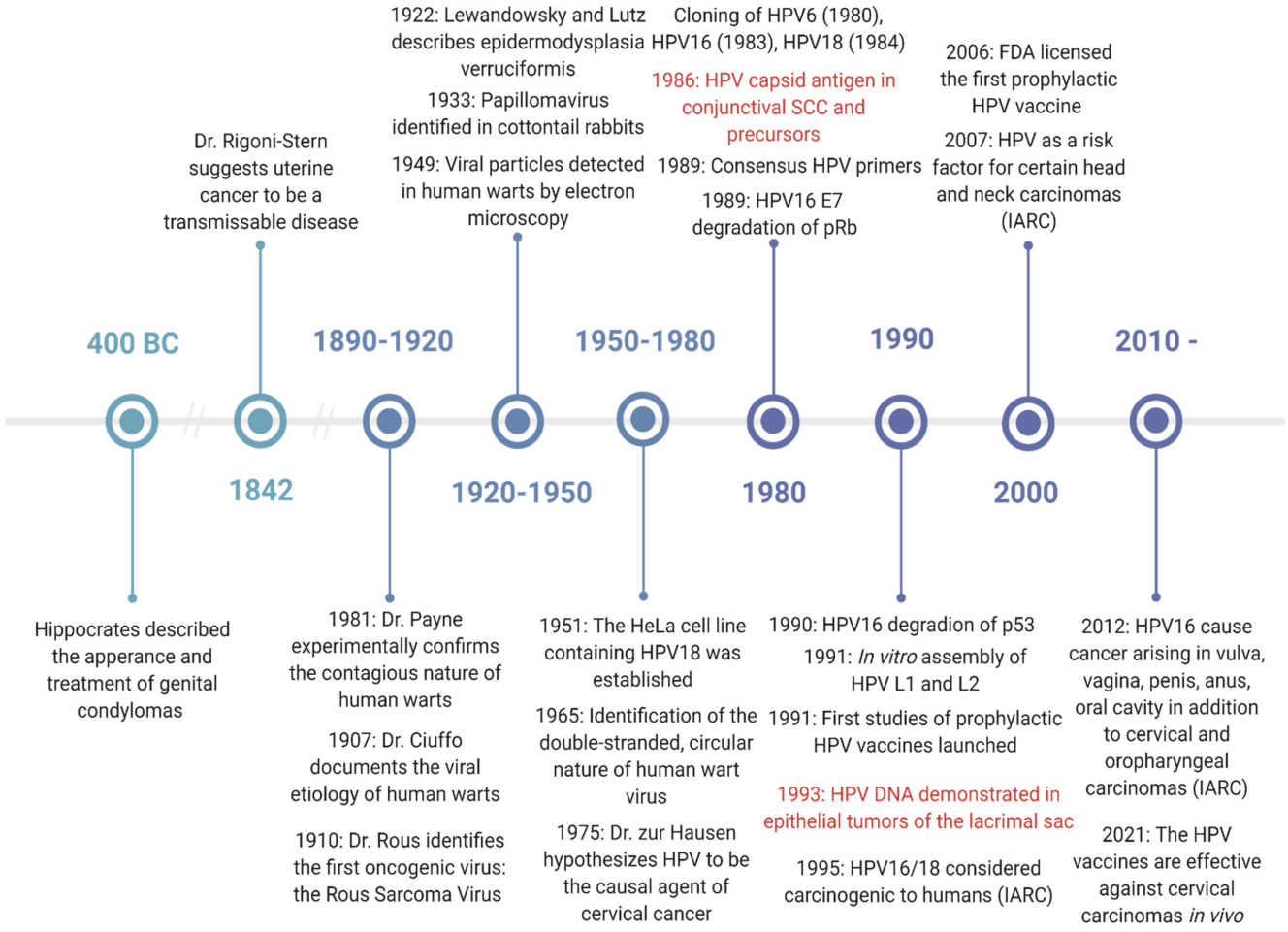

FIGURE 1 A timeline illustrating important aspects in the history of human papillomavirus (HPV) and their implication in cancer. Events regarding HPV in ocular adnexal tissue is marked in red. IARC: International Agency for Reseach on Cancer. The figure is created with Biorender.com.

the host cell replication and may initially be stimulated by the wound healing process (Ozbun, 2019).

In the productive stages of an HPV infection, the viral DNA is kept in a circular, episomal (extrachromosomal) state. Latent infections do not express E6/E7 (Evans et al., 2014). During productive infection, the expression of the viral proteins is closely regulated within the distinct epithelial layers. Highly replicative, productive infections express low levels of E6/E7 confined to the basal epithelial layers, dramatically shifting to a whole-thickness E6/E7 expression combined with low levels of HPV DNA in abortive infections (Evans et al., 2014; Isaacson Wechsler et al., 2012). Deregulation of E6/E7 and loss of contact inhibition, eventually fueled by viral integration, can cause progression of the neoplasia and malignant transformation (Groves & Coleman, 2018; Isaacson Wechsler et al., 2012). Viral integration is centered around specific hotspots in the human genome, often in proximity to fragile chromosomal sites that are susceptible to the insertion of foreign DNA (Pett & Coleman, 2007). In the viral genome, integration breakpoints often occur within the transcription regulatory genes *E1* and *E2* leading to deletion or truncation, causing deregulation of *E6* and *E7* (Evans et al., 2014). Also, disruption of *E1* and epigenetic modulation can cause increased E6 and E7 expression (Evans et al., 2014; Mac & Moody, 2020). Viral integration causes cellular-viral fusion transcripts that have increased stability compared to viral transcripts alone (Jeon & Lambert, 1995; Parfenov et al., 2014), causing higher E6 and E7 expression levels than those with episomal HR-HPV (Parfenov et al., 2014). The integrational process fuels the cell's transforming activity (Symer et al., 2022; The Cancer Genome Atlas Research Network, 2017), but simultaneously the productive infection passes on to an abortive one and is no longer transmissible. Hence, the integrational process is unfortunate for the host cell and the viral particle. Of note, malignant transformation may occur prior to viral integration and may rely on an alternative HPV E2/E4/E5-driven carcinogenesis, which seems to be especially evident for a subset of head and neck SCC (HNSCC) (Ren et al., 2020; Speel, 2017).

The HPV E6 and E7 oncoproteins

The E5, E6, and E7 genes have evolved differently in different epithelial niches and can explain the broad variability of pathogenic effects and tropism of different HPV genotypes (Egawa et al., 2015). The properties of E6 and E7 are best understood in HPV16/18, and are therefore described in the context of these two genotypes.

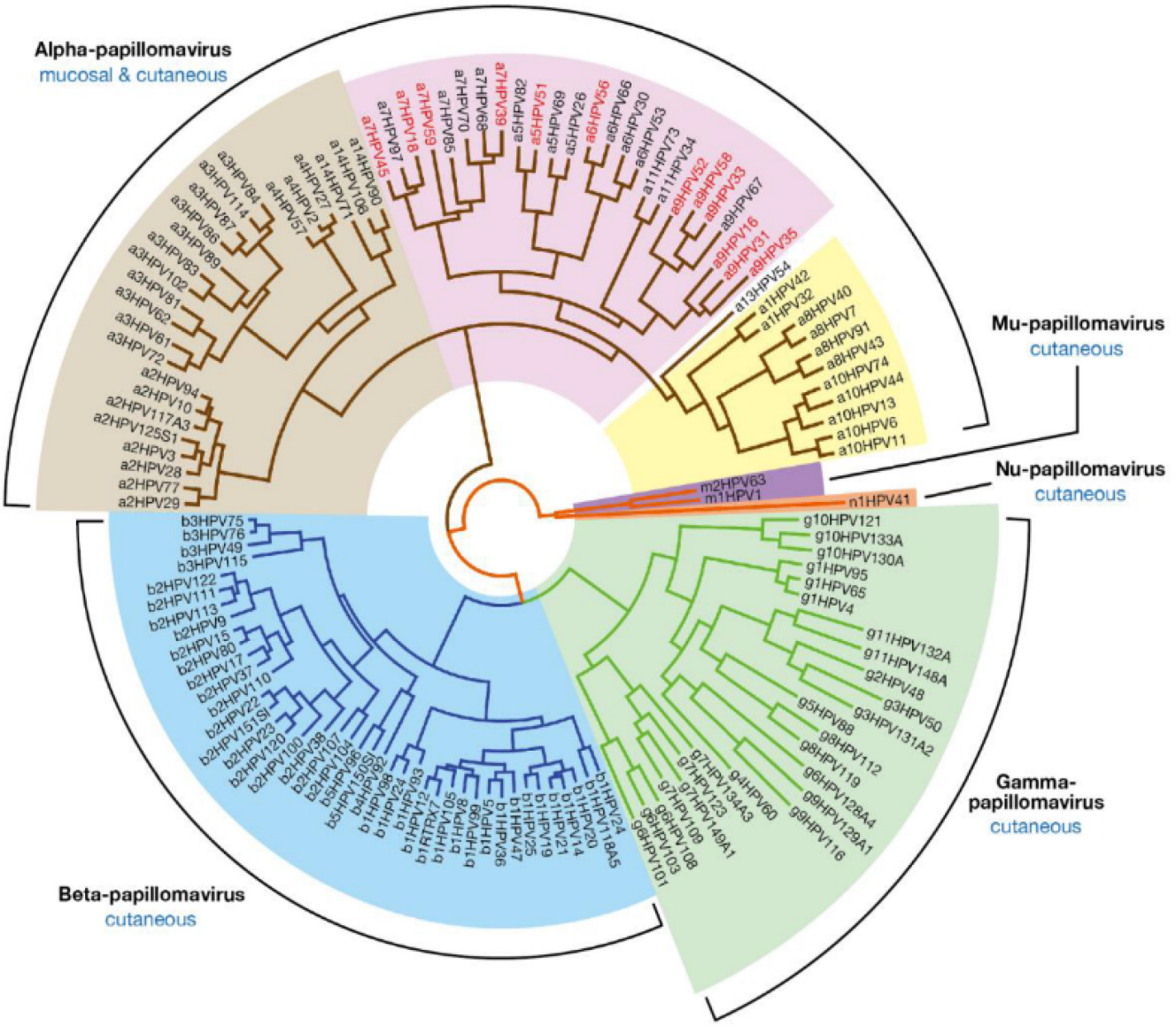

FIGURE 2 The phylogenetic tree of the human papillomaviruses shows the evolutionary relationship between human papillomaviruses as of 2015. More genotypes have now officially been established, and by 1 September 2021 the total number of HPVs is 228. Genotypes that are considered carcinogenic in cervical cancer ("high-risk genotypes") by the International Agency for Research on Cancer are written in red. The figure is from Egawa et al., Human Papillomaviruses; Epithelial Tropisms, and the Development of Neoplasia. Viruses (2015):7:3863–3890 and is published under the CC-BY 4.0 license (Egawa et al., 2015).

Compelling evidence shows that combined *and* maintained E6/E7 expression is necessary for transformation and maintaining a malignant phenotype of HPV-related carcinomas (International Agency for Research on Cancer, 2012b; Jabbar et al., 2009; Yamato et al., 2008), and that the inhibition of these two genes causes resumed p53 and pRb function, directing the cell for cellular senescence or apoptosis (Butz et al., 2000; von Knebel Doeberitz et al., 1992). To create a cellular environment suitable for HPV replication, the HPV oncogenes alter a broad range of host cell targets. Actually, the viral oncogenes E6 and E7 can induce all of the six original "hallmarks of cancer" described in the seminal work by Hanahan and Weinberg (2000), and recently reviewed in the context of HPV (Pal & Kundu, 2019). The two most well-documented effects of E6 and E7 are the degradation of p53 (by E6) and the retinoblastoma protein by ubiquitination (by E7), causing evasion of the two, probably most important, growth suppressors of the cell. In the event of DNA damage or other types of intrinsic cellular stress, the p53 is activated and pursues its functions within growth arrest and DNA repair – or directs the cell towards cellular senescence or apoptosis. Briefly, the blockage of p53 leads to uncontrolled entry through the G1 and G2 checkpoints of the cell cycle, increasing the risk of somatic mutations and genomic instability. Further destabilization of the cell cycle is caused by the degradation of pRb and its associated pocket proteins p107 and p130, which normally bind the E2F proteins that initiate transcription of cellular proteins required for cell division (Figure 3) (Weinberg, 1995). Hence, uncontrolled access to a "pseudo" S phase of the cell cycle is gained, even in differentiated keratinocytes in the suprabasal epithelial layers. E7 is also the main contributor to induce chromosomal instability (Duensing et al., 2000). E6 and E7 are further capable of deregulating the intrinsic and extrinsic pathway of apoptosis (e.g., by degradation of the pro-apoptotic proteins Bax

and Bak), immortalizing keratinocytes through telomerase activation, deregulating the innate and adaptive immune system, inducing angiogenesis and epithelial-to-mesenchyme transition, and acquire sustained proliferative signaling (Pal & Kundu, 2019). However, further description of these features lays outside the scope of this thesis.

Despite the degradation of central anti-tumorigenic checkpoints and vital structures of cellular integrity by HPV, the effects of HPV alone are *not* sufficient for inducing a persistent infection and acquiring a malignant phenotype, which requires further genetic/epigenetic events. This is clearly illustrated by the prevalence of HPV infection that far exceeds the number of HPV-related carcinomas (Chesson et al., 2014). Failure to clear the infection causes persistency of the infection – a prerequisite for malignant transformation (Stensen et al., 2016). Other factors contributing to HPV-related transformation are related to the virus, the host, or the local microenvironment at the site of infection and the pathogenesis is most likely multifactorial (Egawa et al., 2015).

HPV vaccines

Today, three different prophylactic HPV vaccines are available: Cervarix (HPV16 and 18), Gardasil (HPV6, 11, 16, and 18), and Gardasil9 (HPV6, 11, 18, 31, 33, 45, 52, and 58). The vaccines are made with recombinant DNA technology based on the capsid-forming L1 protein to induce specific antibodies blocking viral entry. The vaccines produce specific immune responses, higher than those acquired through natural infection (Beachler et al., 2016), and yield high efficacy (Kjaer et al., 2021; Lei et al., 2020). The vaccines were developed to protect from carcinomas of the uterine cervix, their precursor lesions, and genital condylomas but have proven effective in preventing HPV-related disease of several anatomic locations affected by the genotypes included in the vaccines (Martínez-Gómez et al., 2019). Although designed to prevent viral entry, studies applying adjuvant vaccination to treat existing HPV-positive tumors have shown a moderate efficacy in reducing the risk and time to recurrence. This is especially evident in low-grade intraepithelial neoplasia and papillomas which still harbor an episomal state of the viral particle and thereby intact L1 expression (Garbuglia et al., 2020; Ghelardi et al., 2018; Kang et al., 2013; Rosenberg et al., 2019; Swedish et al., 2012).

Methods for HPV detection

There is a wide variety of methods for HPV detection with differences in sensitivity, specificity, availability, reproducibility, workload, and costs (Table 1).

p16 encoded by the *CDKN2A* gene is a tumor supressor protein that is not expressed under homeostatic conditions. p16 expression is a marker of transforming HPV infections in cervical and oropharyngeal carcinomas (tonsillar and base of tongue), serving as an important diagnostic biomarker included in the AJCC TNM staging (Figure 3) (Panwar et al., 2017; von Knebel Doeberitz et al., 2012). However, p16 expression is not specific to HPV infection and may increase in other circumstances (e.g., by loss-of-function variants in *RB1*). Minichromosome maintenance (MCM) and Ki67 are other proteins regulated by pRb that may act as surrogate markers of HPV E7 protein expression (Ikenberg et al., 2013; Tornesello et al., 2013), but are not specific for such lesions.

Causality of HPV

The detection of HPV DNA in a specimen does not imply that the virus is the causative agent of cancer development but may instead be a matter of correlation. The ultimate tool to determine causality, the performance of a randomized controlled trial (RCT), would be completely unethical in this setting. Therefore, integration of diverse type of evidence from observational studies must be used to evaluate a potential causal relationship, which was conceptualized by the Bradford Hill criteria in 1965 (Hill, 1965), and further elaborated by Rothman et al. (2008) and Vandenbroucke et al. (2016) among others. On this basis, the IARC has concluded a causal relationship between certain HPV and cervical carcinoma (International Agency for Research on Cancer, 1995), and later on expanding this to include subsets of carcinomas arising in the oropharynx, especially in tonsillar and base of tongue SCC, oral cavity, penis, vagina, vulva, and anus (International Agency for Research on Cancer, 2012).

The next question is whether this causal relationship can be applied to other HPV-associated tumors where the strength of association is less clear. Both the conjunctiva and the lacrimal drainage system (LDS) are colonized with a broad range of commensal pathogens (Ali, 2021), and viral presence in these locations could be a matter of correlation. In the 90th volume of the IARC monographs from 2007, the role of HPV in conjunctival carcinomas was briefly discussed, and it was concluded that the evidence was inadequate, mainly due to limited and inconsistent data (International Agency for Research on Cancer). The role of HPV in other epithelial tumors of the ocular adnexa was not mentioned. The present thesis will discuss the role of HPV in epithelial neoplasms of the ocular adnexal tissue, taking the advances in recent years into account.

Anatomy and histology of the ocular adnexal tissue

Mucosal epithelium covers the ocular surface (the cornea, the corneal limbus, and the conjunctiva) and the lacrimal apparatus. Both the mucosal linings of the conjunctiva and the lacrimal sac are derived from the embryonic surface ectoderm. The conjunctival epithelium at the palpebral part consists of stratified columnar epithelium that gradually thickens towards the fornices along with a significant increase of mucous-producing goblet cells (Figure 4). At the

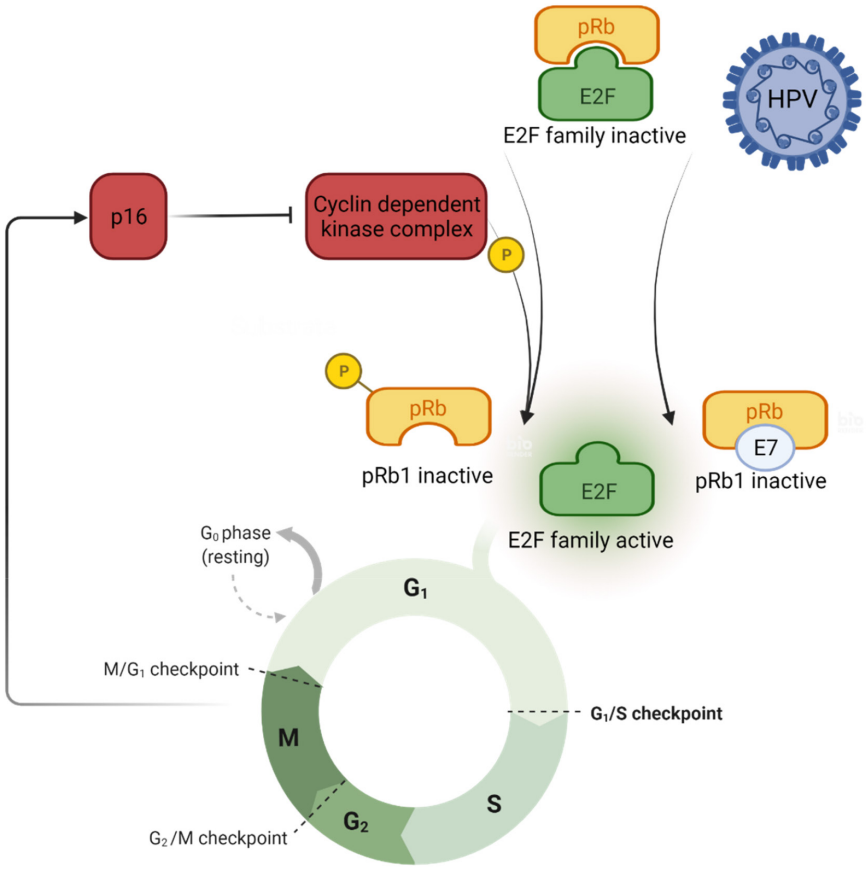

FIGURE 3 Inactivation of pRb, by phosphorylation or inactivation by HPV E7, activates the transcription factor family E2F at the G_1/S checkpoint of mitosis. The progression of the cell cycle leads to a compensatory expression of the tumor suppressor p16. The figure is created with BioRender.com.

corneal limbus, the epithelium is stratified squamous, containing niches of the important limbal stem cells, which are believed to give rise to the majority of conjunctival CIN and SCC (Gichuhi et al., 2014). The underlying *substantia propria* of the conjunctiva consists of highly vascularized connective tissue containing nerves, lymphatics, and resident leukocytes, including lymphocytes, mast cells, plasma cells, and histocytes. At some locations, the lymphocytes are organized in follicles and make up the conjunctiva-associated lymphoid tissue (CALT). The lymphatic vessels drain to the lymph nodes in the preauricular, submandibular, and deep cervical regions. The bulbar conjunctiva is separated from the sclera by the fibrous tissue of the Tenon's capsule.

The conjunctiva forms a physical and immunological barrier to protect the globe from noxious agents and pathogens. Furthermore, the conjunctiva plays a vital role in lubricating the ocular surface by contributing to the tear film production by the interspersed mucin-producing goblet cells – a prerequisite for maintaining the transparency of the cornea and allowing frictionless movement of the eye. Cellular debris, pathogens, and dust are removed from the conjunctiva by the tear flow through the lacrimal puncta leading into the lacrimal drainage system (LDS) upon blinking. From there, the tears drain into the nasal cavity below the inferior meatus. The stratified squamous epithelium that covers the canaliculi and the upper part of the lacrimal sac changes to a pseudostratified/stratified columnar epithelium in the remaining part of the sac and the nasolacrimal duct. Hence, the first part is continuous with the conjunctival epithelium, whereas the lower part is continuous with the respiratory epithelium. Both the conjunctiva and the LDS are theoretically susceptible to HPV infection for various reasons:

1. The stratified (squamous) mucosal epithelium,
2. The direct exposure to the environment (conjunctiva) and indirectly by the tear flow (lacrimal drainage system) in which HPV particles can be transferred,
3. The presence of epithelial transition zones at the corneal limbus and in the lacrimal sac.

The proximity between the conjunctiva and lacrimal drainage system – in developmental, anatomical, histological, and functional aspects – makes it reasonable to investigate the tumors arising from these locations together in the search for shared pathogenesis.

Conjunctival intraepithelial neoplasia and carcinoma

Conjunctival intraepithelial neoplasia (CIN) includes all degrees of conjunctival dysplasia and SCC in situ, all classified as benign tumors. The tumors are generally slow-growing and are initially expanding within the epithelium. Subgroups of conjunctival CIN harbor

TABLE 1 Comparison of different methodologies for human papillomavirus detection, with sensitivity and specificity profiles calculated for oropharyngeal squamous cell carcinomas

Method	Sensitivity (95% CI)	Specificity (95% CI)	Advantages	Disadvantages
p16 IHC	83.3 (69.0–91.8)[a]	93.5 (88.4–96.5)[a]	- Fast	- Inter- and intraobserver variability
			- Inexpensive	- Surrogate marker
			- Broad availability	- Lacking consensus of cut-off thresholds
			- Serve as an independent prognostic marker[a]	- Not validated for conjunctival or LDS tumors
HPV DNA PCR	90.4 (81.4–95.3)[a]	81.1 (71.9–87.8)[a]	- Sensitive	- May detect "passenger infections"
			- Broad availability	- Stromal cell contamination
				- Expensive
				- Time-consuming
				- Easily contaminated
				- Requires primers and thereby a pre-analytical knowledge about the flanking region of interest.
HPV DNA ISH	81.1 (71.9–87.8)[a]	94.9 (79.1–98.9)[a]	- Morphological information	- Inter- and intraobserver variability
				- Signals may be few/weak
			- Specific	- Low sensitivity, especially in low viral burden
HPV mRNA ISH	93.1 (87.4–96.4)[a]	91.9 (78.8–97.2)[a]	-Morphological information	- Inter- and intraobserver variability
			- Transcriptionally active HPV	- High workload
			- Signal amplification yields a high sensitivity	

Note: Data marked with red are based on the available literature calculated from conjunctival squamous cell carcinomas.

Abbreviations: CI, confidence intervals; DNA, deoxyribonucleic acid; HPV, human papillomavirus; IHC, immunohistochemistry; ISH, in-situ hybridization; LDS, lacrimal drainage system; mRNA, messenger ribonucleic acid; NA, not applicable; p16, tumor suppressor protein 16; PCR, polymerase chain reaction; RT-PCR, reverse-transcriptase polymerase chain reaction.

[a]Based on a meta-analysis by Jakobsen KK, Carlander AF, Bendtsen S, et al. 2021: Diagnostic accuracy of HPV detection in patients with oropharyngeal squamous cell carcinoma: A systematic review and meta-analysis. *Viruses*. 2021:13(9):1692.

malignant potential and grow invasively into the underlying *substantia propria* or Bowman's layer and become *per definition* an invasive SCC. The term *ocular surface squamous neoplasia* (OSSN) encompasses pre-invasive and invasive squamous conjunctival neoplasia (Lee & Hirst, 1995) but is not recommended by the American Joint Committee on Cancer for final diagnosis (Amin et al., 2017a, 2017b).

Epidemiology and demographics

Conjunctival SCC is the most common malignant tumor of the ocular surface worldwide, with a reported increase in incidence (Chokunonga et al., 2013; Gichuhi et al., 2013; Wabinga et al., 2014). There is a marked variation in incidence by geographical region, with a steep increase towards the equator (Gichuhi et al., 2013; Newton et al., 1996). The highest incidence is reported in Sub-Saharan Africa (e.g., Botswana, Namibia, Mozambique, and Malawi), with age-adjusted incidence rates ranging between 2.92–3.65 per 100 000/year (Hämmerl et al., 2019), representing a public health challenge in these countries. In temperate regions (e.g., Denmark, Canada, and the USA), reported population-based incidence rates are 0.02–0.045 per 100 000/year (Darwich et al., 2020; Ramberg et al., 2015; Sun et al., 1997). Based on these geographical differences, two disease patterns of conjunctival SCC become apparent: In temperate regions, conjunctival SCC is a disease affecting primarily Caucasian men in their seventh decade (Kao et al., 2012; Ramberg et al., 2015; Sun et al., 1997), in contrast to the marked female predominance with a lower median age at diagnosis (third to the fourth decade) in Sub-Saharan countries (Gichuhi et al., 2013, 2015; Hämmerl et al., 2019; Merz et al., 2019; Ruffieux et al., 2021; Tiong et al., 2013).

Risk factors

The location of the conjunctiva, directly exposed to the external environment, makes it vulnerable to a broad range of carcinogens, including ultraviolet radiation (UVR), pathogens, petroleum chemicals, and cigarette smoke – all proposed risk factors for conjunctival SCC development (Gichuhi et al., 2015, 2016b; Napora et al., 1990). Furthermore, endogenous factors such as immunosuppression, DNA-repair deficiencies (e.g., due to the genetic disorder *xeroderma pigmentosum*), chronic ocular inflammation, and vitamin A deficiency are reported risk factors for the disease (Gichuhi et al., 2014). The high incidence of conjunctival SCC in Sub-Saharan countries coincides with two important risk factors for conjunctival SCC development: high exposure to UVR and a high prevalence of people living with human immunodeficiency virus (HIV).

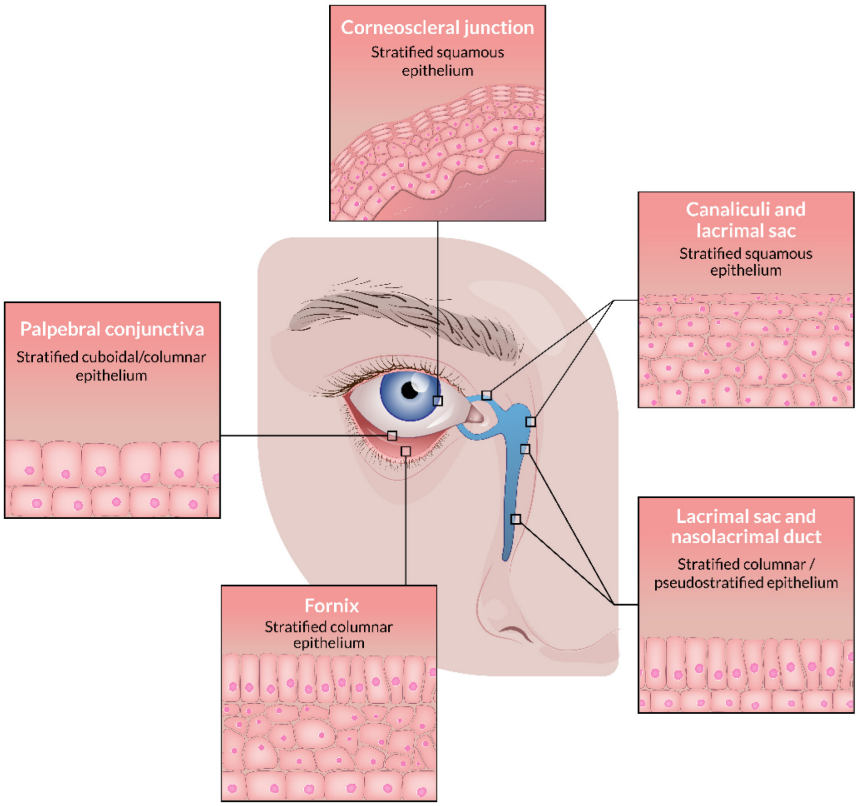

FIGURE 4 The epithelia of the conjunctiva and the lacrimal drainage system from Ramberg I, Heegaard S. Human Papillomavirus Related Neoplasia of the Ocular Adnexa. Viruses. 2021. 13:1522. Published under the CC-BY license.

Ultraviolet radiation

Ultraviolet radiation (UVR) is a human carcinogen able to induce, maintain, and drive cancer development (International Agency for Research on Cancer, 2012a). UVR causes direct DNA damage in keratinocytes by rearranging the covalent bonds between consecutive pyrimidines, leading to the creation of pyrimidine 6–4 photoproducts (6–4 PPs) and cyclobutene pyrimidine dimers (CPDs), preferably at CpG sites of the genome (Brash, 2015). The accelerated deamination of cytosine when it is part of a CPD causes the specific DNA signature of UV radiation: >60% of the variants being C>T transitions and>5% being tandem CC>TT transitions (Brash, 2015; Pfeifer et al., 2005).

Ecological and mechanistic evidence support UVR as a risk factor for conjunctival SCC development. The reverse relationship between latitude, used as a proxy for solar UVR exposure, and the incidence of conjunctival SCC has been shown in several studies (Gichuhi et al., 2013; Newton et al., 1996; Sun et al., 1997). Newton et al. (1996) described a double in incidence in every 10° decrease in latitude towards the equator. Such studies are, however, prone to confounding, since the exposure on an individual level is unknown. Nevertheless, the findings from these ecological studies are supported in case–control studies using questionnaires and occupational history (e.g., outdoor occupation versus office work) as indications of UV radiation exposure (Lee & Hirst, 1997; Napora et al., 1990; Newton et al., 2002). Indirect measures of UVR exposure are used due to the inherent difficulties of measuring the exposure to UVR on an individual level.

Evidence supporting UVR-induced mutagenesis in conjunctival SCC is also found at clinical, histopathological, and molecular levels. Conjunctival SCC most frequently arises in the nasal limbus – the conjunctival part that receives the highest amounts of ambient UVR. Series from the USA ($n = 60$), Thailand ($n = 30$), and Australia ($n = 288$) have reported that 30%–50% of the patients synchronously presented with other ocular diseases related to UVR exposure: pterygium, pinguecula, solar elastosis, or nuclear sclerosis (Lee & Hirst, 1997; Tulvatana et al., 2003; Tunc et al., 1999). A few studies have investigated the genomic profile of conjunctival SCC and the precursor lesions (Galor et al., 2016; Lazo de la Vega et al., 2020; Ramos-Betancourt et al., 2020). One study reported a UVR mutational signature in 10 out of 10 conjunctival CIN investigated by whole-exome sequencing (Ramos-Betancourt et al., 2020). A few studies have reported C>T and CC>TT variants in selected genes, such as *TP53* (Ateenyi-Agaba et al., 2004) and the *TERT* promoter (Scholz et al., 2015; Starita et al., 2018), indicative of UVR-induced damage; however, caution must be paid when deducing the mutagen from genetic patterns in few loci.

Immunosuppression

According to the Joint United Nations Programme on HIV/AIDS (UNAIDS), the worldwide prevalence of people living with HIV was 37.6 million in 2020 (Joint United Nations Programme on HIV/AIDS, 2021), with more than 50% living in Sub-Saharan Africa, where the estimated HIV prevalence exceeds 10% (Joint United Nations Programme on HIV/AIDS, 2021). Impaired

immune surveillance increases the risk of conjunctival CIN and SCC development and it is estimated that one-third of all conjunctival SCC cases are due to HIV (Hämmerl et al., 2019). A cross-sectional cohort study from South Africa reported a 21.5 (95% CI 16.3–28.4) higher OR of conjunctival SCC in people living with HIV compared to people without HIV (Dhokotera et al., 2019). Interestingly, this study was performed in the era of antiretroviral therapy (ART) and is, along with several other studies, implying that the incidence of conjunctival SCC continues to rise despite ART treatment (Dhokotera et al., 2019). A retrospective case series from India reported conjunctival CIN or SCC as the first presenting symptom of an HIV infection in one-fourth of the patients (Kaliki et al., 2017). People living with HIV have a higher prevalence of conjunctival SCC than the ocular AIDS-defining diseases Kaposi sarcoma and non-Hodgkin's lymphoma; however, it is not (yet) considered an AIDS-defining disease (Rogena et al., 2015). Immunosuppression also increases the risk of developing UVR-induced tumors (International Agency for Reseach on Cancer 2012). The incidence peak of conjunctival SCC in Sub-Saharan Africa is probably due to the high prevalence of both risk factors, which act independently or synergistically to increase the incidence of the disease.

HIV-related conjunctival SCC is associated with bilateral tumors with a more aggressive clinical course, higher risk of recurrence, and higher risk of lymph node and distant metastasis developing at an earlier age (De Silva et al., 2005; Gichuhi et al., 2013, 2016b; Kamal et al., 2015; Merz et al., 2019; Muchengeti et al., 2021). Therefore, it is recommended to perform HIV testing in patients presenting with conjunctival CIN or SCC in an HIV-endemic area or when patients present with an atypical course of the tumor, including disease debut under the age of 40 (Gichuhi et al., 2016b). Suppression of the immune system from other causes (e.g., iatrogenic immunosuppression in autoimmune diseases or after organ transplantation) also increases the risk of conjunctival SCC development (Lazo de la Vega et al., 2020; Shields et al., 2011). This underlines the important role of the immune system in these tumors.

Human papillomavirus

The first report of HPV in conjunctival CIN and SCC was published in 1986 by McDonnell et al. (1986)demonstrating HPV capsid antigen in the tumor tissue by immunoperoxidase (Figure 1). With the advent of polymerase chain reaction (Mullis et al., 1986) and the heat-stable Taq DNA polymerase, the presence of HPV DNA in conjunctival CIN and SCC was later shown by the same group (McDonnell et al., 1989). In the following years, the reported prevalence of HPV in conjunctival CIN and SCC has been substantially variable (0%–100%) (Guthoff et al., 2009; Kuo et al., 2006; Manderwad et al., 2009; Scott et al., 2002), creating uncertainty of the role of HPV in these tumors. A recent work by our group addressed this controversity by systematically evaluating the literature and performing a meta-analysis to explore different sources of heterogeneity (Ramberg et al., 2020a). Based on 1410 patients with conjunctival CIN and SCC, the pooled prevalence of HPV-positive tumors was 26% (95% CI 0.16–0.36) with HPV16 as the most prevalent genotype, while the HPV prevalence was 1% (95% CI 0.0–3.0, I^2 =62%) among controls, most frequently the low-risk HPV11. The pooled OR of HPV in conjunctival CIN and SCC compared to control tissue was 8.2 (95% CI 3.70–19.16) but with considerable heterogeneity (I^2 =62.0, 95% CI 38.1%–75.5%). By meta-regression analysis, geography was revealed as a statistically significant contributor to the observed heterogeneity (50.4%) (pooled OR = 1.74, 95% CI 0.85–3.54, I^2 =0% in African studies compared to pooled OR = 5.82–44.32, I^2 =50% in other countries, p = 0.013). The heterogeneity between studies using PCR as the single method was higher than studies using a combination of methods (I^2 =71%, p <0.01 versus I^2 =11%, p = 0.35), but the effect size was not significantly different (OR 6.21, 95% CI 1.93–19.99 versus OR 14.57, 95% CI 3.39–62.56, p = 0.52). Different studies applied different types of tissue in the control group, including healthy conjunctival tissue, pinguecula, inflamed conjunctiva, pterygium, and "minimal dysplasia", which may alter the effect size, since some of these conditions also have a reported association to HPV. The estimated effect size using healthy conjunctival tissue was OR 18.51 (95% CI 3.77–90.94), and studies using diseased conjunctiva had an OR of 5.08 (95% CI 1.93–13.34, p = 0.18). Also intriguingly, no HPV-positive cases among the controls were reported in studies combining HPV DNA PCR with another detection modality. Taken together, these data suggest that HPV is associated with a subset of conjunctival CIN and SCC, but also revealed gaps in our understanding of HPV in these tumors; are these HPV infections biologically relevant? What are the characteristics of the HPV-positive conjunctival CIN and SCC and do they differ from the HPV-negative counterparts? And, is HPV a potential biomarker for future diagnostics and prognostication of these tumors?

Clinical characteristics

Conjunctival CIN and SCC in situ have overlapping clinical characteristics with their invasive counterparts as well as other non-neoplastic conjunctival pathologies (e.g., pterygium, pinguecula, vitamin A deficiency, pannus, dyskeratoses, and pyogenic granuloma) and neoplastic conjunctival pathologies (e.g., papilloma, nevus, [amelanotic] malignant melanoma, sebaceous gland carcinoma, Kaposi sarcoma). The tumor is usually unilateral and positioned in the interpalpebral fissure near the corneal limbus, often involving the peripheral cornea. It often presents as an elevated, relatively sharply demarcated tumor with prominent tortuous and dilated tumor-feeding vessels. The tumor may have a papillomatous, nodular, leukoplakic, or gelatinous appearance. The color varies according to the vascularity of the tumor from light grey to red and may even appear highly pigmented, the latter often being present in Africans (Gichuhi et al., 2013). A prospective multicenter study of conjunctival lesions from

Kenya, which included 496 patients, reported a positive predictive value of 54% by using the clinical appearance alone to detect conjunctival CIN and SCC, making an assessment based on clinical findings alone insufficient for proper diagnosis (Gichuhi et al., 2015). A more recent systematic review estimated the clinical diagnostic accuracy to be 84% (Siedlecki et al., 2016), still leaving histopathological examination as the gold standard for diagnosis.

Histopathology and staging

Based on the thickness of the affected epithelium by cellular atypia, conjunctival CIN is graded into grade I/mild: cellular atypia covering 1/3 of the epithelium, grade II/moderate: cellular atypia within 2/3 of the epithelium, and grade III/severe: cellular atypia covering >2/3 of the epithelium but with a preserved layer of superficial maturation (Amin et al., 2017a, 2017b). Conjunctival SCC in situ is defined as full-thickness atypia. The tumor is classified as an invasive SCC if the tumor breaches the basal membrane and is no longer confined to the epithelium. Conjunctival SCC is TNM (tumor, node, metastasis) staged in accordance with the eighth edition of the American Joint Committee on Cancer (AJCC) staging manual (Amin et al., 2017a, 2017b). A representative biopsy (incisional or excisional) is a prerequisite for tumor staging, since staging requires an assessment of the tumor in relation to the basal membrane. The accuracy of excisional biopsy is high, with an estimate of 0.98 (range 0.97–0.98) (Siedlecki et al., 2016). The risk of recurrence and metastasis correlates with advanced T stage (Chauhan et al., 2014; Galor et al., 2012; Yousef & Finger, 2012) but cannot predict response to the initial treatment (Bellerive et al., 2018; Parrozzani et al., 2017; Shields et al., 2013). No prognostic factors other than TNM classification are incorporated into the staging algorithm.

Histopathologically, the tumors are graded based on the degree of cellular atypia (Amin et al., 2017a, 2017b). The assessment of atypia is based on the degree of pleomorphism, the nuclear-to-cytoplasm ratio, the number of (atypical) mitoses, and the presence of squamous features such as intercellular bridges. Poorly differentiated conjunctival SCC may present a basaloid morphology with comedo necrosis and scarce cytoplasm. The tumor might appear pigmented due to the inclusion of dendritic melanocytes (Shields et al., 2008). Histologically, the tumor exhibits loss of polarization and the presence of mitoses above *stratum basale*. An inflammatory response is often present in the *substantia propria*.

By histochemistry and immunohistochemistry, the tumors often express pan cytokeratin (AE1/AE3), p63, p53, and the proliferation marker Ki67 (Grossniklaus et al., 2018; Jakobiec et al., 2013). The tumor is often negative in PAS due to loss of epithelial goblet cells (Jakobiec et al., 2013). Increased expression of PD-L1 is reported in 47%–100% of conjunctival SCC, with increased expression in advanced-stage disease (> AJCC stage T2) (Nagarajan et al., 2019; Wolkow et al., 2019).

Genetic profile

Frequent pathogenic variants in *TP53* and the *TERT* promoter have been reported in conjunctival CIN and SCC (Ateenyi-Agaba et al., 2004; Scholz et al., 2015; Starita et al., 2018). However, the genomic *profile* of conjunctival SCC has only been addressed in one previous study (Lazo de la Vega et al., 2020), and two additional studies have performed whole-exome sequencing of pre-invasive tumors (Galor et al., 2016; Ramos-Betancourt et al., 2020).

In HPV-negative (or undetermined HPV status) conjunctival CIN and SCC, frequent alterations have been detected in *CDKN2A*, *SOX2*, *MYC* (primarily copy number variations [CNVs]), *TP53* (primarily somatic variants), *PIK3CA*, *NOTCH1*, and *RB1* (even distribution of CNVs and somatic variants) (Lazo de la Vega et al., 2020). Loss of heterozygosity in *CDKN2A* and *TP53* were evident also in the pre-invasive tumors, indicating that these are early events in conjunctival SCC development (Lazo de la Vega et al., 2020). In the two studies assessing the genetic profile of conjunctival CIN, frequent variants in cancer-related genes were detected in *TP53*, *HGF*, and *CREBBP* (Galor et al., 2016; Ramos-Betancourt et al., 2020). As for now, no genomic biomarker has been incorporated into conjunctival CIN and SCC diagnostics, prognostication, or prediction. The genetic profile of HPV-positive conjunctival CIN and SCC has not been reported in the literature.

Treatment and prognosis

Conjunctival CIN and SCC can be treated with a broad range of modalities, including surgical excision, topical/intralesional pharmacotherapy, and brachytherapy, applied as monotherapy or in combination. To date, there are no randomized controlled trials (RCT) investigating surgical versus medical treatment modalities (Gichuhi et al., 2013), and according to the clinical trial registration site clinicaltrials.gov no such studies are currently planned. One RCT has shown a decrease in recurrence after the application of adjuvant 5-fluorouracil (5-FU) in invasive carcinomas (Gichuhi et al., 2016a), and observational studies support a similar benefit from adjuvant interferon alpha-2b (INF-α2b) (Siedlecki et al., 2016).

Conjunctival SCC is locally destructive rather than metastatic and is usually considered an indolent and low-grade malignancy (Kaliki et al., 2017). However, advanced stage conjunctival SCC (> AJCC T2) has a severe prognosis regarding eye-sparing treatment and has a higher risk of recurrence, metastasis, and disease-specific death (McKelvie et al., 2002; Miller et al., 2014). Also, conjunctival SCC in immunocompromised patients presents with more aggressive tumors. Even though the tumor rarely disseminates, reported predilection sites are the preauricular, submandibular, and cervical lymph nodes and salivary glands, lungs and bone by hematogenous spread (McKelvie et al., 2002; Tabbara et al., 1988). The risk of recurrence is highest within the first year of diagnosis; however, it remains high for 5 years, whereafter the risk declines (Lee & Hirst, 1997; Miller et al., 2014; Ramberg et al., 2015). The recurrence risk is reported to increase in positive surgical

margins, larger lesions (Lee & Hirst, 1997), tarsal or palpebral locations of the tumor (Nanji et al., 2014).

The main challenge in the treatment of conjunctival CIN and SCC is the high rates of recurrence, both in surgically and medically treated patients. Also, non-responsiveness and intolerance to topical pharmacotherapy and the underlying molecular factors for this treatment failure are to date unknown, leaving a gap in our understanding of these tumors' pathogenesis.

Epithelial neoplasia of the lacrimal drainage system

Tumors arising from the LDS are rare; however, the precise incidence and geographical distribution are unknown. LDS neoplasias are most often malignant tumors of epithelial origin (carcinomas) (Krishna & Coupland, 2017; Marthin et al., 2005). A total of 539 cases of lacrimal sac carcinomas were identified in a literature search between 1960 and 2020: most commonly SCC and non-keratinizing SCC (previously transitional cell carcinomas), and rarely mucoepidermoid carcinomas, adenocarcinomas, and adenoid cystic carcinomas (Ramberg et al., 2020b). Exceedingly rare, malignant oncocytoma, lymphoepithelial carcinoma, and sebaceous carcinoma of the LDS present in the LDS (Ramberg et al., 2020b). A chart review from Denmark reported that tumors constituted 4.5% of all LDS lesions that were histopathologically evaluated (Marthin et al., 2005). Primary malignant neoplasms of the LDS constitute approximately 1% (range 0%–3.5%) of all samples collected during dacryocystorhinostomy (DCR) for presumed primary acquired nasolacrimal duct obstruction (PANDO) (Koturovic et al., 2017). Most histological subtypes have an equal gender distribution or a slight male predominance while adenoid cystic carcinomas have a reported female predominance (Ramberg et al., 2020b). The reported median age at diagnosis is 58 years across different histological subtypes (Ramberg et al., 2020b).

Risk factors for LDS carcinoma development

The pathogeneses of epithelial tumors of the LDS are poorly elucidated. Suspected risk factors include chronic inflammation, human papillomavirus, and LDS papillomatosis (Ramberg et al., 2020b). Squamous cell papilloma is the most common *benign* neoplasm of the LDS. The growth pattern can be exophytic, endophytic, or mixed, and the morphology can be transitional, squamous, or oncocytic. Histologically, an exophytic papilloma consists of one pedunculated or several papillomatous processes with a core of vascularized connective tissue with an overlying acanthotic epithelium. In endophytic papillomas, the papillomatous tissue expands within the epithelium, but still respects the basal membrane. Sir Duke-Elder stated in 1974 that "the two common epithelial tumors of the lacrimal sac – papillomata and carcinomata – have been generally considered as separate entities although it has always been agreed that the former are liable to assume malignant characteristics at any time in their evolution" (Duke-Elder & MacFaul, 1974). However, from the time these words were written, we are still unaware of the factors leading to malignant transformation or recurrence of these histologically benign tumors.

A few case reports and small case series have investigated the presence of HPV DNA within LDS *papillomas* in a total of 20 cases (Ramberg & Heegaard, 2021), most frequently the low-risk HPV6 and 11 (Buchwald et al., 1996; Jones et al., 2020; Madreperla et al., 1993; Nakamura et al., 1997; Sjö et al., 2007, Vickers et al., 2010). In the neighboring sinonasal cavity, HPV is implicated in exophytic papillomas, and is also a suspected contributor to malignant transformation of sinonasal inverted papillomas, although there is still significant controversy on this topic (Lawson et al., 2008; Stepp et al., 2021).

Five series have investigated the presence of HPV DNA in LDS SCC ($n = 30$), being positive in 73% (Afrogheh et al., 2016; Hongo et al., 2022; Madreperla et al., 1993; Sjö et al., 2007). The most commonly detected genotype is the high-risk HPV16 (Ramberg et al., 2020b). HPV transcripts have been investigated in two studies; the first failed to document HPV RNA in HPV DNA-positive cases (Sjö et al., 2007), while the other detected HPV transcripts in six out of seven cases (Hongo et al., 2022). Based on these reports, HPV may be a contributor to carcinoma development in the LDS, calling for further research.

Clinical characteristics

Malignant tumors of the LDS are often unilateral with origin in the lacrimal sac. The usual symptomatology of an LDS tumor is described to exhibit three clinical phases: The first phase simulates simple dacryocystitis or -stenosis with epiphora and rarely purulent or bloody discharge (Duke-Elder & MacFaul, 1974). It is not uncommon that this initial phase lasts several years. In a literature review by our group, patients presenting with epiphora had a median duration of 18 months (range 1–180 months) until diagnosis (Ramberg et al., 2020b). In the next phase, an actual tumor formation presents as a firm, irreducible mass above the medial canthal tendon. The passage of fluid may be possible upon syringing; however, sanguineous reflux during the procedure is a warning sign. These two initial stages are not specific to LDS neoplasia, making the diagnosis of an LDS malignancy notoriously challenging. This shared symptomatology is shown in series evaluating the histopathology of biopsies from DCR taken during routine DCR for presumed PANDO: Malignant tumors constitute approximately 1% of all cases, and a specific pathology constitutes approximately 6% (Koturovic et al., 2017). Eventually, the tumor may develop into the ulcerative third phase with a visible extension of the tumor (Yamato et al., 2008).

Prognosis

The rareness of the tumors, the various histological subtypes, and the lack of established grading and treatment regimens make it difficult to conclude the prognosis of these tumors. Furthermore, most data to date have a retrospective set-up with variable and limited follow-up of the patients. Nevertheless, the majority of the tumors present with advanced-stage disease at diagnosis with frequent development of distant metastases.

AIMS AND HYPOTHESES

The overall aim of the studies included in the present thesis was to investigate the role of HPV in the development of conjunctival SCC, carcinomas of the LDS, and their potential precursor lesions. We further aimed to correlate the HPV status to clinical, histopathological, and genomic features to define characteristics of the HPV-positive and -negative geno- and phenotype.

Based on these aims and the knowledge of HPV-positive carcinomas to date, we defined the following hypotheses:

(I) Alpha-genus HPV is associated with conjunctival intraepithelial neoplasia and carcinoma and epithelial neoplasms of the lacrimal drainage system.
(II) The clinical profile (age and gender distribution) of the tumors differs based on HPV status.
(III) The high-risk HPV-positive tumors harbor pathognomonic histological features including koilocytotic atypia, a basaloid, non-keratinizing morphology, and p16 overexpression distinct from their HPV-negative counterparts.
(IV) The high-risk HPV-positive carcinomas harbor a genetic profile distinct from their HPV-negative counterparts, including wild-type status of *TP53*.
(V) HPV status has prognostic value in conjunctival SCC, LDS carcinomas, and their precursor lesions.

MATERIAL AND METHODS

Patient and tumor material

Patients diagnosed with conjunctival intraepithelial dysplasia (I), carcinoma in situ (I, II), squamous cell carcinoma (I, II), or an epithelial neoplasm of the lacrimal drainage system (III) were identified by searching the Danish Pathology Register and a manual review of the pathology files at the former Eye Pathology Institute, University of Copenhagen, Denmark (Figure 5). Patient information and tumor characteristics were retrieved from the pathology files, medical records (when available), and the Danish Register of Causes of Death.

Corresponsing formalin-fixed and paraffin-embedded (FFPE) tissue blocks were retrieved from local pathology departments, followed by validation of the diagnoses. The conjunctival CIN and SCC were staged according to the eighth edition of the American Joint Classification on Cancer (AJCC) staging manual (Amin et al., 2017a, 2017b). The LDS neoplasms were classified according to the fourth edition of the WHO classification of tumors of the eye (Heegaard et al., 2018). No AJCC staging manual exists for carcinomas of the lacrimal drainage system.

Discussion of patients and tumor material

The largest limitation in these studies is the small sample sizes resulting from the rarity of these tumors. Therefore, the included number of patients was not based on an *a priori* power calculation, but instead we included all cases identified in the database searches. The small sample sizes means that most analyses probably are underpowered, increasing the risk of type II errors.

The patients included in the present thesis are identified using two different sources: the pathology files from the former Eye Pathology Institute (ØPI), University of Copenhagen, Denmark, and the nationwide Danish Pathology Register. The ØPI had eye pathology expertise receiving samples from hospitals and private ophthalmologists throughout Denmark for routine diagnostics free of charge. The pathology files from this center were manually reviewed to identify the patients. To detect samples not referred to the ØPI, we searched the Danish Pathology Register using the systemized nomenclature of medicine (SNOMED). The Danish Pathology Register contains data from 1970, and since it became mandatory to register all surgical pathology specimens in 1997, the register has a nearly 100% coverage (Bjerregaard & Larsen, 2011). We used codes for primary, recurrent, and metastatic intraepithelial neoplasia, papilloma, and carcinoma combined with the topographical codes for conjunctiva, cornea, caruncle, and the lacrimal drainage system. According to Danish practice, all patients have a histopathological diagnosis prior to topical treatment and have a record in the pathology databases, but cases not submitted to the ØPI before 1997 may have been missed.

The Danish Cause of Death Registry contains data regarding the time of death and the underlying cause of death on all deceased Danish residents (Helweg-Larsen, 2011). The registered underlying cause of death is dependent on the correctness of the individual physician's registration, since the autopsy rate is declining (Helweg-Larsen, 2011). The registry is therefore applicable for monitoring all-cause mortality rather than disease-specific mortality (Helweg-Larsen, 2011).

Due to the retrospective setup, patient inclusion is always prone to bias. By including all cases identified in a nationwide cohort, we consider the risk of referral bias as low. However, excluding samples with sparse material for ancillary testing may have created a selection bias towards larger lesions. Also, decalcification of osseous tissue with formic acid led to exclusion of advanced-stage LDS carcinomas.

Methods

Literature review (IV)

Literature searches

Literature searches were performed in three different medical search engines: MEDLINE via PubMed,

Embase via Ovid, and Scopus. The search strategy combined free text searches and medical subjects headings (Appendix A1).

Histopathology and immunohistochemistry (I, II, III)

Five-µm sections from each FFPE block were stained with hematoxylin and eosin (HE) following a standard procedure. The sections were reviewed by an eye pathologist (SH) and the author to confirm the diagnosis and evaluate the tumor content for downstream molecular analyses.

Immunohistochemistry (IHC) is an easy and fast way to evaluate single antigens while simultaneously obtaining morphological information. Four-µm sections were mounted on coated slides (Dako). The analyses were performed on a Ventana Benchmark Ultra (Ventana Medical Systems, AZ, USA) using the UltraView/Optiview detection kit (760–500/760–700) for evaluation of the following antigens: p16 (I, II, and III), human epidermal growth factor-2 (HER2)(III), and androgen receptor (AR)(III), an by using the Dako Autostainer Link 48 (Agilent, CA, USA) for CK-ACEAM (III). Nuclear and cytoplasmic staining in >75% of the cells were considered p16 positive. HER2 expression was evaluated according to ASCO guidelines (Wolff et al., 2013). An overview of the clones and manufacturers is provided in Table 2.

DNA extraction (I, II, III)

DNA was extracted for HPV DNA PCR analysis and DNA sequencing. The tumor tissue was macrodissected to enrich for tumor cells. Three to five unstained FFPE sections were transferred to an Eppendorf tube for DNA extraction. We applied a DNA extraction method developed for FFPE tissue (Gene Read DNA FFPE kit #180134, Qiagen, Hilden, Germany) using the QIAcube (Qiagen) and quantified the DNA using Qubit DNA HS assay kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). In study II and III, a DNA level of 250 ng was set as the lower input limit due to an observed quality decrease of the sequencing data with lower DNA inputs.

Human papillomavirus DNA polymerase chain reaction (I, II, III)

To investigate the presence of HPV DNA within the samples, we performed HPV DNA PCR. All cases were submitted to PCR for the housekeeping gene GAPDH using the GAPDH-A/GAPDH-B primers (Table 3) to ensure the integrity of the DNA. If sufficient, the samples were submitted to HPV DNA PCR using the consensus primers GP5+/6+ (Table 3). The amplicons were visualized by gel electrophoresis. Each electrophoresis included a pool of HPV-positive tumors as a positive control and H_2O as a negative control. All HPV-positive amplicons were genotyped by sequencing. Sequencing libraries were constructed using the KAPA HTP Library Preparation Kit (Kapabiosystems; Roche Diagnostics, Basel, Switzerland). After ligation of adaptors (NEXTflex-96 DNA barcodes; Bioo Scientific, Austin, TX, USA) and repair of blunt ends, the prepared library was quality checked by automated electrophoresis (TapeStation; Agilent, Santa Clara, CA, USA) by the High Sensitivity D5000 kit from Agilent. Also, a quantitative evaluation of the purified library was performed using the Qubit dsDNA BR Kit (Invitrogen) on a Qubit Fluorometer. Paired-end sequencing was performed on an Illumina platform (MiSeq, San Diego, CA, USA). The HPV reference genome (HPV_REF) from https://pave.niaid.nih.gov was used to map the reads.

HPV E6/E7 gene expression (I, II, III)

To evaluate the transcriptional activity of HPV E6 and E7 and simultaneously obtain morphological information,

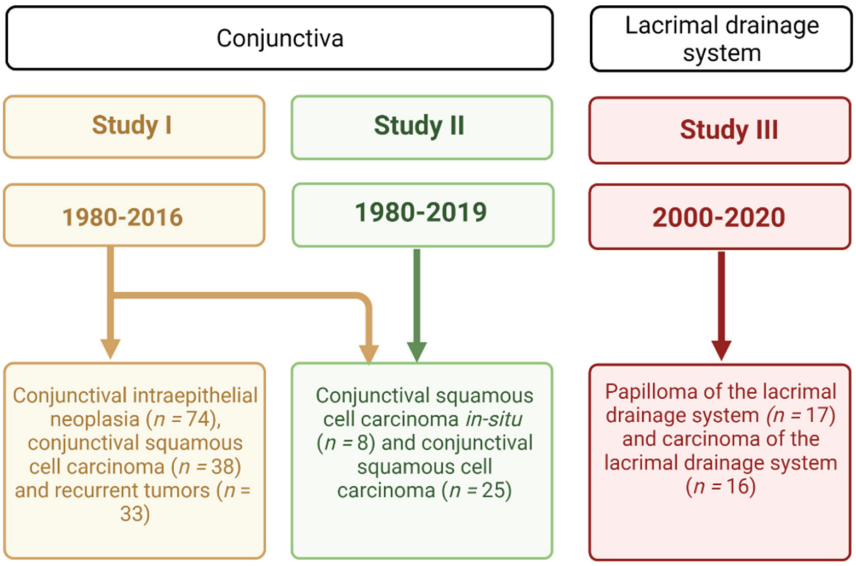

FIGURE 5 An overview of the patient material included in the present thesis.

the cases were submitted to chromogenic mRNA in-situ hybridization (mRNA ISH). Tumor samples with an intact expression of the housekeeping gene cyclophilin B (PPIB) were included for E6/E7 mRNA ISH. A negative control (the bacterial gene diaminopimelate [DapB]) was also applied to determine non-specific expression. The analysis was performed using the automated RNAscope VS Reagent Kit (Advanced Cell Diagnostics, Newark, CA, USA) on a Ventana Discovery ULTRA staining module (Ventana Medical Systems). We applied the high-risk HPV18 probe covering the high-risk genotypes 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, and 82 and the low-risk HPV6 probe covering the HPV6, 11, 40, 42, 43, and 44 (Advanced Cell Diagnostics). A semi-quantitative scoring algorithm recommended by the manufacturer was applied (grade 0: no staining or <1 dot per 10 cells; grade 1: 1–3 dots per cell; grade 2: 4–9 dots per cell; grade 3: 10–15 dots per cell and < 10% of dots were in clusters, grade 4: >15 dots/cell and > 10% of dots were in clusters) (Advanced Cell Diagnostics, 2017).

High-throughput DNA sequencing (II, III)

A total of 33 cases of conjunctival SCC in situ and SCC and 12 cases of LDS carcinomas were eligible for DNA sequencing analysis. The analysis was performed at two different time points but followed the same procedure. The library was prepared using the TruSight Oncology 500 Library Preparation Kit from Illumina following the manufacturer's instructions (TSO500 Reference Guide, document number 1000000067621, Illumina) with a DNA input of 250 ng. This is a hybrid capture-based sequencing method suitable for FFPE tissue that targets 523 cancer-related genes (Appendix A2). Paired-end sequencing (2x150 base pairs) was performed on a NovaSeq 6000 sequencing platform (Illumina).

The bioinformatic analysis was performed at the Center for Genomic Medicine at Rigshospitalet, Copenhagen, Denmark. The files were converted from . bcl files to FASTQ files (bcl2fastq v2.16.0.10, Illumina), and quality was assessed with the fastQC v.0.11.85 (Andrew, 2010). Sequencing adaptors, low-quality reads, and other technical reads were removed with BBduk v.38.26. The human reference genome hg19 was used to realign the reads. Mutect2 from the Genome Analysis Toolkit package (Broad Institute, Cambridge, MA, USA) was used for variant calling following their best practices for tumor-only short somatic variant detection (van der Auwera & O'Connor, 2020). The Genome Aggregation Database v.2.1.1 (Karczewski et al., 2020), based on more than 140 000 individuals, was used as a germline source, since we did not include matched samples of normal tissue in the analyses.

Annotation was performed using the Ingenuity Variant Analysis (Qiagen Bioinformatics, Redwood City, CA, USA). The filtering criteria are provided in the appendix (A3). The Integrative Genomics Viewer v.2.5.2 was used to inspect the reads in the context of adjacent reads. Different databases of somatically acquired variants in cancer were used for interpretation of the annotated variants: the COSMIC database (Catalogue of Somatic Mutations in Cancer) (Tate et al., 2019), cBioportal (Cerami et al., 2012; Gao et al., 2013), and OncoKB (Chakravarty et al., 2017).

Statistical analyses (I, II, III)

All statistical analyses were performed using R v.3.6.1. A comparison of groups with continuous variables (e.g., age) was performed by an unpaired t-test assuming equal variances and a normal distribution. A comparison of frequencies with categorical variables was performed with a chi-square test or, if a cell in the contingency table included a value below 5, a Fisher exact test (Altman, 1991). Logistic regression adjusted for age and sex was used to estimate differences in clinical and histopathological variables between HPV-positive and HPV-negative conjunctival CIN and SCC. Rate of recurrence and all-cause mortality were calculated with age- and sex-adjusted Cox regression model. In the recurrence calculations, death was treated as a competing risk. A p-value below 0.05 was considered statistically significant.

Discussion of methods

The quality of the molecular analyses is highly dependent on the input. Therefore, great efforts were put into histological evaluation of the specimens to choose the most optimal tumor block and ensure sufficient tumor content. The following precautions were taken to yield the best possible quality of the IHC and ISH sections: sectioning was performed shortly before analysis, stored in a refrigerator until use, and positive and negative controls were applied on each section as suggested on the datasheets (Bass et al., 2014). Eventually, after sectioning for the molecular analyses, an HE-stained section was evaluated to ensure the presence of tumor cells. Macro dissection guided by an HE-stained section further increased the tumor content for downstream analyses.

TABLE 2 An overview of immunohistochemical analyses

Antibody	Clone	Manufacturer	Dilution	Staining module	Study
p16	E6H4	Roche	RTU	Ventana	I, II, III
CK-ACEAM	CM162C	Biocare	1:50	Dako	III
AR	AR441	Dako	1:100	Ventana	III
HER2	4B5	Roche	RTU	Ventana	III

Abbreviation: RTU, Ready to use.

TABLE 3 The primers used for quality control of DNA (GAPDH-a/b) and human papillomavirus (HPV) DNA detection (GP5+/6+)

PCR primer	Sequence
GAPDH-a	5′-GAAGGTGAAGGTCGGAGTC-3′
GAPDH-b	5′-GAAGATGGTGATGGGATTTC-3′
GP5+	5′TTTGTTACTGTGGTAGATACTAC-3′
GP6+	5′-GAAAAATAAACTGTAAATCATATTC-3′

Formalin-fixed and paraffin-embedded tumor tissue

FFPE of tumor tissue is the most common type of tissue preservation at pathology departments worldwide, and the abundance and availability have led to substantial efforts to optimize advanced molecular analyses to achieve valid results from FFPE tumor tissue. Accordingly, we have applied a DNA extraction method designed explicitly for FFPE that contains uracil-DNA-glycosylase. This enzyme removes FFPE-induced deaminated cytosine (resulting in uracil) and reduces the amount of artificial C>T variants in PCR and sequencing reactions (Do & Dobrovic, 2012). Furthermore, the TSO500 library preparation kit for FFPE tissue was applied in the DNA sequencing studies (II and III). The kit includes unique molecular identifiers (UMIs) that make initial library amplification by PCR unnecessary, thereby increasing the validity of the sequencing data (Smith et al., 2017). Nevertheless, the number of usable sequencing reads is decreased when using FFPE compared to fresh frozen tissue, and low-frequency sequencing artifacts must be expected (Meldrum et al., 2011).

Investigations of human papillomavirus

The sensitivity and specificity of methods for HPV detection vary considerably (Table 1). Also, there is a broad heterogeneity in the HPV prevalence of especially conjunctival CIN and SCC in the literature. Therefore, we applied a multimodal approach for HPV testing, combining HPV DNA PCR as the most commonly detected method with E6/E7 mRNA ISH that evaluates the expression of the HPV E6/E7 oncoproteins. While the PCR analysis may amplify biologically irrelevant HPV DNA ("passenger infection"), specific E6/E7 mRNA expression patterns strongly correlate with biologically relevant infections (Evans et al., 2014; International Agency for Research on Cancer, 2012b). We used the consensus GP5+/6+ primers for HPV DNA PCR, that targets conserved regions of the L1 region. However, the primers are only detecting sequences that are partially homologous and will therefore not detect viral sequences that are distantly related. Evaluation of p16 was included due to the importance of this biomarker in cervical and certain head and neck carcinomas.

Intra- and interobserver variability

The IHC and mRNA ISH analyses are prone to bias due to inter- and intraobserver variability. The IHC sections were evaluated independently by SH and the author to decrease the risk of bias. In case of disagreement, a consensus was reached between the authors. Furthermore, the scoring algorithm described above was followed to increase transparency and enhance the reproducibility of the results.

Targeted high-throughput DNA sequencing

The genomic profiles of conjunctival and LDS carcinoma are largely unknown. Only one study has previously reported the genomic profile of conjunctival SCC using targeted DNA sequencing, but this study did not evaluate the genomic aberrations based on HPV status (Lazo de la Vega et al., 2020). Therefore, we aimed to perform a broad, sensitive DNA sequencing suitable for FFPE. We chose a targeted high-throughput sequencing method that allows deep sequencing depth, even in fragmented FFPE DNA input. However, we did not investigate copy number variations, structural aberrations, and mutational signatures but restricted the analysis to single-nucleotide variants, insertions, and deletions (indels), and therefore only provide insight to parts of the genomic aberrations of these tumors. Another downside of the TSO500 library preparation kit is the high amounts of required input DNA. Although the manufacturer suggested a 30-ng input, we experienced a considerable decrease in the quality of the reads below 250 ng, causing the exclusion of many samples. These are the first studies of their kind and were performed without a matched normal tissue sample to correct for germline status. Therefore, we have applied conservative criteria in the filtering to avoid spurious calls, which may have overlooked potentially important variants. On the other hand, all included samples achieved good quality reports even in the oldest samples with a high coverage: a median coverage of 633X and 770X in studies III and II, respectively, and 98%–98.9% of the reads above 50X.

One could argue that a whole-exome sequencing (WES) approach would have been better because we did not have an *a priori* knowledge of the relevant genes. However, when these studies were initiated, fresh frozen tissue with paired normal tissue was required for WES analysis, and a prospective collection of specimens would take decades. Also, the achieved sequencing depth is lower in WES than in a targeted approach within a reasonable economic framework. Therefore, we chose a highly reproducible method suitable for FFPE tissue restricted to known cancer-promoting genes.

Ethics

This thesis adheres to the tenets of the Declaration of Helsinki version 10, 2013, for medical research involving human subjects (World Medical Association Declaration of Helsinki, 2013). The studies were approved by the Danish Data Protection Agency (RH-2013-30-1035, 02288) and the Scientific Ethics Committee of the Capital Region of Denmark (H-16044879, 55 827).

RESULTS

The main results from studies I, II, and III are summarized below. The results of study IV are included in the introduction and the thesis discussion.

Epidemiology

A total of 154 primary conjunctival CIN and carcinoma and 39 recurrences were detected in Denmark between 1980–2016. The age-adjusted incidence rate increased from 0.03/100000 (95% CI 0.002–0.12) to 0.12/100000 (95% CI 0.045–0.25) with an annual average increase of 3.9% (95% CI 2.0–5.8, $p < 0.05$). From 2000–2020, 31 patients with 33 tumors were diagnosed with a papilloma ($n = 17$) or a carcinoma ($n = 16$) of the LDS.

Clinical characteristics

A total of 112 primary conjunctival CIN and SCC and 33 recurrent tumors were included to assess the association to HPV (I). There was a male predominance (81 out of 112, 72%) and a mean age of 67.5 years in men (standard deviation [SD] 13.3) and 61.9 years in women (SD 17.3) at diagnosis. A location at the bulbar conjunctiva, primarily located at the corneal limbus, was the most common. Tumors in the conjunctival fornix was more likely an invasive carcinoma tumors located to the epibulbar conjunctiva (OR 11.5, 95% CI 3.5–46.1, $p = 0.0001$). Demographics and tumor characteristics are presented in Table 4.

Patients with an LDS papilloma were younger at diagnosis compared to patients with an LDS carcinoma (mean difference 12.9 years, 95% CI 3.0–22.8, $p = 0.012$). A male predominance was detected among patients with LDS papillomas (14 out of 18, 63%) and carcinomas (10 out of 16, 63%) (OR 5.2, 95% CI 0.6–53.0, $p = 0.14$).

Human papillomavirus

Prevalence of HPV

Twenty-four out of 112 (21%) primary, treatment naïve conjunctival CIN and SCC were HPV positive by HPV DNA PCR in our cohort (I). The genotypes detected were HPV16 ($n = 18$), HPV11 ($n = 2$), HPV6 ($n = 2$), HPV33 ($n = 1$), and HPV39 ($n = 1$). Only high-risk HPV16 was detected in the invasive carcinomas. All HPV DNA-positive tumors were expressing E6/E7 HPV transcripts, except one case. In the negative case, p16 expression was also lacking. Recurrences from an HPV-positive primary tumor were all HPV positive. In the LDS, 12 out of 15 (80%) papillomas and 10 out of 15 (67%) SCC were HPV positive by HPV DNA PCR (HPV11, 16). All PCR-positive cases (excluding one case that failed in the pre-analytical phase) expressed HPV E6/E7 mRNA throughout the epithelium, while all HPV-negative cases lacked E6/E7 expression. No expression was detected in the surrounding stromal tissue or the HPV DNA negative cases.

Clinical features of HPV-positive squamous cell carcinomas and precursors

Results from study I imply that the clinical characteristics of HPV-positive conjunctival SCCs differ from the HPV-negative conjunctival SCCs. Patients with an HPV-positive tumor were significantly younger at diagnosis compared to those with HPV-negative tumors (mean difference 11.5 years, 95% CI 5.2–17.9 years, $p = 0.0005$). Among females, a history of cervical dysplasia was associated with an HPV-positive tumor (OR 18.9, 95% CI 1.4–1120, $p = 0.0098$). A high (≥3) Charlson morbidity index was significantly associated with an HPV-negative tumor (OR 0.08, 95% CI 0.035–0.28), although not considering the age difference between the groups. The risk of recurrence was higher among HPV-positive tumors (hazard ratio [HR] 2.30, 95% CI 1.02–5.21, $p = 0.046$). No differences were detected in the all-cause mortality when adjusting for age and sex (HR 0.54, 95% CI 0.23–1.31, $p = 0.17$).

Among the neoplasms arising in the LDS (III, $n = 31$), no statistically significant differences were detected between HPV-positive and HPV-negative tumors regarding gender or age at diagnosis. Due to the limited sample size, survival analyses were not performed.

Histopathological features of HPV-positive squamous cell carcinomas and precursors

The cases of conjunctival CIN or SCC presented with keratinization (66%) and a highly inflammed stromal tissue (83%) (Figure 6). Conjunctival tumors exhibiting a non-keratinizing morphology were more likely to be HPV positive (OR 13.5, 95% CI 4.1–53.8, $p < 0.0001$). In conjunctival SCC, p16 expression predicted high-risk HPV infection with a sensitivity of 90% and a specificity of 66% using HPV DNA PCR as reference. The sensitivity increased to 100% when using E6/E7 mRNA ISH as a reference. In LDS carcinomas, the sensitivity was 100% and the specificity 77%.

Genomic features of squamous cell carcinomas of the conjunctiva and lacrimal drainage system

Thirty-three cases of conjunctival SCC ($n = 8$ in-situ carcinomas and $n = 33$ invasive carcinomas, Figure 7) and 13 carcinomas of the LDS (Figure 8) were included for genomic analyses (II and III). These included $n = 19$ HPV-positive carcinomas ($n = 15$ HPV16, $n = 1$ HPV39, $n = 3$ HPV11) and $n = 26$ HPV-negative carcinomas using HPV E6/E7 expression as reference. The most mutated genes were *TP53*, *KMT2D*, *PIK3CA*, *CDKN2A*, and *RB1* in the conjunctival SCC and *PIK3CA*, *BCR*, *TGFR2*, *FGFR3*, *PIK3R1*, and *TP53* in the LDS carcinomas. By analyzing the pre-invasive conjunctival SCC, it appeared that pathogenic variants in *TP53*, *CDKN2A*, *PIK3CA*, and *RB1* were early events in the carcinogenic transformation of conjunctival SCC, since these variants were seen in both in-situ and invasive carcinomas. Contrastingly, pathogenic variants in *JAK3*, *FGFR3*, and the KMT family, most frequently *KMT2D* (p.Q2553*, p.Q4284*, p.R5086*, and p.R5432Q), were mostly seen in the invasive carcinomas.

TABLE 4 Clinical and histopathological characteristics of patients with conjunctival intraepithelial neoplasia and squamous cell carcinoma ($n = 112$) and carcinoma of the lacrimal drainage system ($n = 16$) in a Danish cohort

	Conjunctival intraepithelial neoplasia and carcinoma				Carcinoma of the lacrimal drainage system			
	HPV positive $n = 24$	**HPV negative $n = 88$**	**Total $n = 112$**	**Estimate (95% CI), p-value**	**HPV positive $n = 10$**	**HPV negative $n = 6$**	**Total $n = 16$**	**Estimate (95% CI), p-value**
Age (years), mean (SD)	56.9 (13.1)	68.4 (14.2)	65.9 (14.7)	11.5 (5.2–17.9), $p = 0.0005$	61.0 (14.1)	64.8 (16.2)	62.4 (15.0)	3.7 (−13.9–21.4), $p = 0.66$
Gender, *n* (%)								
Male	16 (67)	65 (74)	81 (72)	OR 1.03 (0.4–3.2), $p = 0.65$	7	3	10	OR 2.2 (0.18–29.5), $p = 0.61$
Female	8 (33)	23 (26)	31 (28)	Reference	3	3	6	
Diagnosis, *n* (%)								
Intraepithelial neoplasia	13 (54)	45 (51)	58 (52)		0	0	0	
Carcinoma in situ	5 (21)	11 (13)	16 (14)		0	0	0	
Invasive carcinoma	6 (25)	32 (36)	38 (34)		10	6	16	
P16, positive samples, *n* (%)[b]	18/20 (90)	30/87 (34)	48/107 (45)		7/7	1/6	8/13	
mRNA ISH, positive samples, *n* (%)	18/19 (95)	0/7 (0)	18/26 (69)		9/9	0/4	9/13	
Location, *n* (%)[c]								
Bulbar conjunctiva	16 (67)	73 (83)	89 (79)	OR 1.85 (0.4–5.9), $p = 0.52$[a]	NA	NA	NA	
Tarsal or forniceal conjunctiva	4 (17)	13 (15)	17 (15)	Reference	NA	NA	NA	
Canaliculi	NA	NA	NA	NA	0	0	0	
Lacrimal sac	NA	NA	NA	NA	10	6	16	
AJCC T stage for invasive carcinomas, *n* (%)[c]								
T1	1	6	7		NA	NA	NA	
T2	0	11	11		NA	NA	NA	
T3	2	4	6		NA	NA	NA	
T4	2	2	4		NA	NA	NA	
AJCC N and M stage, *n*	2N, 2M	0	2N, 2M		2N, 2M	1N, 1M	3N, 3M	
Recurrence, *n*	12	21	33	HR 2.30 (1.02–5.21), $p = 0.046$[a]	8	1	9	
All-cause mortality, *n*								
Adjusted				HR 0.54 (0.23–1.31), $p = 0.17$				

Abbreviations: AJCC, American Joint Committee on Cancer; CI, confidence intervals; HPV, human papillomavirus; ISH, in-situ hybridization; M, metastasis; N, lymph node metastasis; NA, not applicable; OR, odds ratio; T, tumor.

[a]HPV-positive tumors compared to HPV-negative tumors.

[b]Only high-risk HPV genotypes are classified as HPV positive, five samples were unavailable for p16 immunohistochemistry due to the lack of tumor tissue.

[c]Data missing.

By stratifying the genomic analysis by HPV status, distinct clusters were detected. The high-risk HPV-positive carcinomas harbored frequent hot-spot variants in *PIK3CA* (*n* = 8 [50%], p.E81K, p.E542K, p.E545K, p.R115L), *FGFR3* (*n* = 5 [31%], p.S249C), and other genes implicated in the PI3K- signaling cascade (*mTOR, TSC1, TSC2, PIK3R1, AKT1, n* = 6). In total, the PI3K signaling was affected in 14 out of 16 (88%) of the high-risk HPV-positive carcinomas. The high-risk positive SCCs from both anatomic locations were characterized by wild-type *TP53* (15 out of 16, 94%), *CDKN2A* (16 out of 16, 100%), and *RB1* (15 out of 16, 94%), in contrast to HPV-negative SCC where *TP53* (22 out of 26, 85%) was the most commonly affected gene. The conjunctival HPV-negative SCC further harbored mutations in *CKDN2A* (7 out of 24, 29%; p.P48L, p.P114L, p.R80*, p.D92fs*28, and p.X148_splice) and *RB1* (6 out of 24, 25%; p.V144Lfs*9, p.V222fs*2, p.W99*, p.V450fs*13, p.X500_splice, p.Q597*, and p.E675*) distinct from their HPV-positive counterparts. The functional impact of the *RB1* variants was confirmed by the overexpression of p16 in all cases. Two p16-positive cases harbored neither high-risk HPV nor *RB1* variants.

DISCUSSION

In the present thesis, we aimed to explore the role of HPV in carcinomas and their precursor lesions arising in the ocular adnexa and its potential impact on clinical, histological, and genetic features in these tumors. Since the first reports of a potential contribution of HPV in conjunctival carcinoma development in the 1980s (McDonnell et al., 1986, 1987, 1989), considerable heterogeneity has been reported in the literature (Ramberg et al., 2020a). The rapid development in molecular diagnostics in recent years has enabled us to apply highly sensitive and specific methods to evaluate the biological relevance of HPV and the, until now largely unexplored, genetic aberrations of these tumors.

While most SCC arising in the uterine cervix are caused by high-risk HPV, 30%–50% of all HNSCCs, dependent on anatomical location, are HPV-driven (Hoffmann & Quabius, 2021). Therefore, the latter provides a unique model for examining the characteristics of HPV-related tumorigenesis compared to other drivers in the same anatomic location (Hoffmann & Quabius, 2021). Accordingly, HPV status serves as an important clinical biomarker of HNSCC regarding clinical (age, gender predilection, lymph node status at diagnosis, and prognosis) and histological (morphology, p16 expression) features – especially in tonsillar and base of tongue SCC (Hoffmann & Quabius, 2021). Stratifying analyses by HPV status have similarly identified clinical and histological differences in ocular adnexal SCC as discussed in the following – although solid evidence is hard to achieve due to the rarity of the tumors.

We have previously reported that 26% (95% CI 16%–36%) of conjunctival CIN and SCC are associated to HPV, using HPV DNA as a reference (Ramberg et al., 2020a). Nagarajan et al., Hongo et al., and Griffin et al. submitted all their cases for E6/E7 mRNA ISH and reported similar frequencies of biologically important infections (26%, 28%, and 36%, respectively) (Griffin et al., 2019; Hongo et al., 2022; Nagarajan et al., 2019), in accordance with study I, which confirmed deregulated HPV E6/E7 expression in all HPV DNA-positive samples except one. In LDS carcinomas, an HPV prevalence ranging from 50%–89% has been reported in the literature (Madreperla et al., 1993; Sjö et al., 2007; Afrogheh et al., 2016; Jones et al., 2020, Ramberg et al., 2022). The biological relevance of these infections was shown with a dysregulated E6/E7 expression pattern in 67% of the LDS carcinomas in study III. As in SCC of the endocervix and the oropharynx, the HPV16 genotype is the main contributor

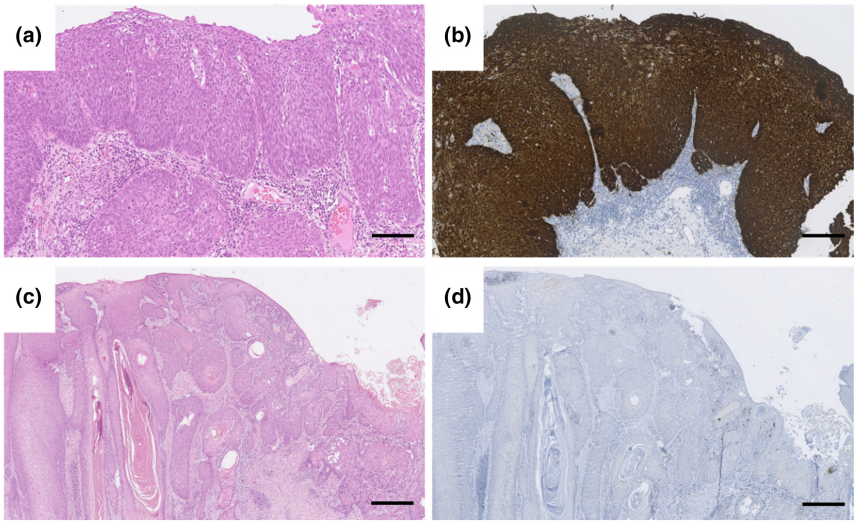

FIGURE 6 Morphology and p16 expression in conjunctival squamous cell carcinoma (SCC). (a) A moderately differentiated, basaloid conjunctival SCC with scant cytoplasm without evidence of keratinization and intercellular bridges on an inflammatory stroma (Hematoxylin–Eosin, scale bar = 150 μm). (b) The tumor exhibit block-positive p16-expression and was HPV16 positive (p16 immunohistochemistry, scale bar = 200 μm). (c) A well-differentiated conjunctival SCC with irregular-sized nests and the presence of large keratin pearls (Hematoxylin–Eosin, scale bar = 300 μm). The tumor was HPV negative by polymerase chain reaction (not shown) and (d) did not express p16 (p16 immunohistochemistry, scale bar = 300 μm).

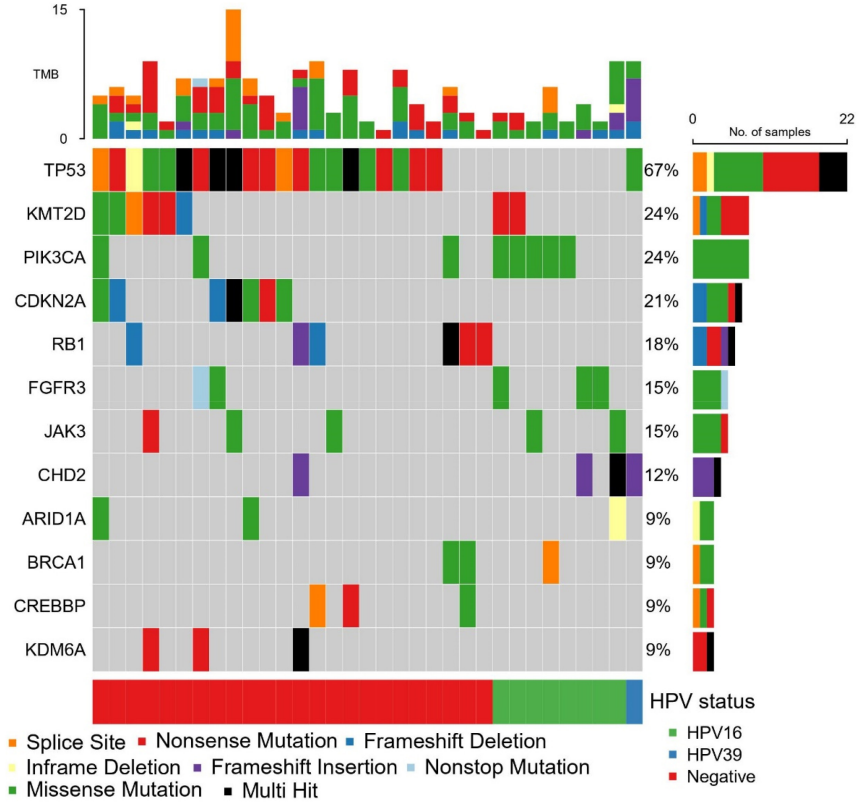

FIGURE 7 The most frequently altered genes in conjunctival intraepithelial neoplasia and carcinoma, stratified by human papillomavirus (HPV) status. From Ramberg I, Vieira FG, Toft PB, von Buchwald C, Funding M, Nielsen FC, Heegaard S. Genomic Alterations in Human Papillomavirus-Positive and -Negative Conjunctival Squamous Cell Carcinomas. Invest Ophthalmol Vis Sci. 2021 Nov 1;62(14):11.

in HPV-positive ocular adnexal carcinomas, with a relatively small palette of other contributing HPV genotypes (Ramberg & Heegaard, 2021).

Clinical features

Few studies have correlated the HPV status of conjunctival SCC and CIN with clinical characteristics (Ramberg & Heegaard, 2021). The HPV-positive patients in our study of conjunctival CIN and SCC were younger at diagnosis compared to their HPV-negative counterparts (Ramberg et al., 2021a). This finding is supported by studies from the USA (Afrogheh et al., 2016) and Japan (Hongo et al., 2022). Regarding gender, an overall male predominance is seen in conjunctival CIN and SCC (in high-latitude countries). However, it is still uncertain if the gender distribution also differs within the HPV subgroups (Afrogheh et al., 2016; Hongo et al., 2022; Ramberg et al., 2021a,). One recent case series investigated the clinical features of the tumors based on HPV status ($n = 31$) and reported a higher AJCC T stage at diagnosis among HPV-positive carcinomas (Nagarajan et al., 2019), which is in accordance with our results. Recently, Hongo et al. (2022) reported a statistically significant association between high-risk HPV-positive tumors and a location in the forniceal or palpebral conjunctiva. In our study of LDS carcinomas (III), we could not detect statistical differences in age and gender based on HPV status. However, the small sample size is a concern in this study.

Histopathological features

Histologically, our analyses of both conjunctival and LDS carcinomas revealed a pathognomonic HPV-induced histological phenotype with a basaloid, non-keratinizing morphology with tumor cells expressing p16, a feature distinct to the well-differentiated HPV-negative carcinomas that more often present with keratinization and absent/low or patchy expression of p16 (Chernock, 2012; Afrogheh et al., 2016; Griffin et al., 2019; Ramberg et al., 2021a; Hongo et al., 2022; Ramberg et al., 2022). Previous reports have identified a lack of HPV E4 expression (Griffin et al., 2019), MCM overexpression (Griffin et al., 2019), and lack of p53 expression in suprabasal layers (Benzerdjeb et al., 2021; Griffin et al., 2019), further indicating phenotypic consequences of HPV (Chernock et al., 2009). The presence of koilocytotic atypia (from Greek "koilos," meaning hollow), hence enlarged squamous cells with dysplastic nuclei and a perinuclear halo, is pathognomonic for the HPV-induced changes in a cell (Koss & Durfee, 1956). The literature on HPV-related conjunctival CIN and SCC has reported highly variable rates of koilocytosis, with sensitivity ranging from 0%–100% and specificity from 63%–100% (Ramberg & Heegaard, 2021), and for now is not a reliable biomarker of HPV in this location.

There is limited data regarding p16 as surrogate for HPV-related conjunctival SCC, and drawing solid conclusions from the available data is complicated by the lack of consensus regarding the definition of

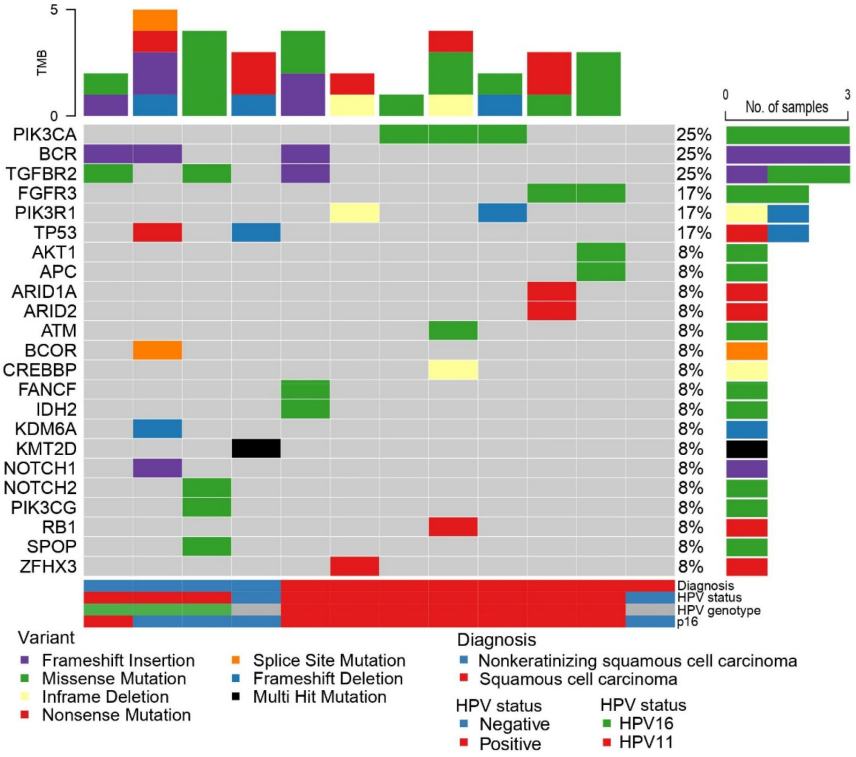

FIGURE 8 The most frequently altered genes in lacrimal sac squamous cell carcinoma, stratified by human papillomavirus (HPV) status. From Ramberg I, Vieira FG, Toft PB, von Buchwald C, Heegaard S. Viral and Genomic Drivers of Squamous Cell Neoplasms Arising in the Lacrimal Drainage System. Cancers 2022, 14, 2558.

p16 overexpression (e.g., dichotomous definition or reporting percentages of positive cells), staining pattern (cytoplasmatic or nuclear staining, or combined), differences in the applied p16 clones (E6H4, 16PO4, and JC8, among others), the reference for HPV positivity (e.g., HPV DNA or RNA), and the method of HPV testing (PCR-based, ISH-based, sequencing, or ELISA). Yet, the sensitivity to detect biologically important HPV infections seems high (Griffin et al., 2019; Ramberg & Heegaard, 2021; Ramberg et al., 2021a; Hongo et al., 2022; Ramberg et al., 2022). The sensitivity of p16 is seemingly higher when E6/E7 mRNA ISH is used as a reference (100%) compared to PCR-based methods (85%–90%), probably due to the detection of "passenger" or E6/E7 independent HPV infections by DNA PCR, while the specificity profiles are comparable (75%–79% using E6/E7 mRNA ISH versus 66%–90% using HPV DNA PCR) (Chauhan et al., 2012; Griffin et al., 2019; Hongo et al., 2022; Ramberg & Heegaard, 2021). p16 expression should not stand alone in determining HPV status, and the recommendation to apply p16 as a single tool to determine HPV status in oropharyngeal SCC by the WHO has been criticized (Amin et al., 2017a, 2017b; Marklund et al., 2020). Instead, p16 expression is useful in combination with HPV DNA PCR to better assess some of the phenotypic consequences of transforming HPV infections. Therefore, a separation between p16-positive/HPV-positive from p16-positive/HPV-negative is crucial for future research on HPV as a biomarker in ocular adnexal SCC, but it requires consensus of p16-expression cut-offs and protocols. Of note, p16 expression serves as a prognostic biomarker for increased survival in oropharyngeal SCC, independent of HPV status (Prigge et al., 2017).

Genomic profile

Based on HPV status, the high-risk HPV-positive SCC harbored a distinct genomic profile compared to the HPV-negative counterparts. Furthermore, the genetic analyses of HPV16-positive SCC of the conjunctiva and the LDS have uncovered striking similarities. The SCCs of both locations seem to be driven by PI3K-AKT pathway activation, most frequently due to the pathogenic, activating variants in *PIK3CA*, especially p.E542K and p.E545K, and *FGFR3*, especially p.S249C, which together were present in 12 out of 15 (80%) HPV16-positive cases. *PIK3CA* is encoding the catalytic subunit of the PI3-kinase. All the variants detected in the present work affect the helical domain of the kinase with known activating downstream effects on the signaling pathway (Litwin et al., 2017), promoting proliferation, cell survival, and cell growth (Figure 9). Pathogenic or likely pathogenic variants in *AKT1, mTOR, TSC1*, and *TSC2* and likely pathogenic, inactivating variants in *PIK3R1* were also detected, and, in total, 14 out of 15 (93%) of these tumors had genetic variants predicted to increase PI3K-AKT signaling. The tumors were further characterized by deregulated high-risk HPV E6/E7 patterns, p16 overexpression, and wild-type status of *TP53*. Probably, a phenotypic loss of p53 is achieved through two different mechanisms: p53 degradation by HPV E6 in the HPV16-positive SCC and genomic variants in the

HPV-negative counterparts. Hence, wild-type status of *TP53* and *RB1* are expected in HPV E6/E7-driven carcinomas. The HPV39 and HPV11 E6 have lower affinity for degradation of p53, and, accordingly, two out of four HPV11- and HPV39-related carcinomas were *TP53* mutant.

Host cell mechanisms may also contribute to HPV-induced carcinogenesis. The Apolipoprotein B mRNA editing catalytic polypeptide-like cytosine deaminases (APOBEC) are upregulated in a cell to assist in viral clearance but, similar to other DNA-editing enzymes, may induce somatic mutations (Chan et al., 2015). The variants have a characteristic 5′TCW mutational motif ("the APOBEC signature") like the *PIK3CA* p.E542K and p.E545K variants observed in the present work. However, it is not possible to deduce the mutagen from mutations in a few loci, but it is a topic for genome-wide studies to come. We can conclude, however, that the genomic profile of HPV16-positive SCCs uncovered in the present work resembles that of HPV-driven carcinomas of other anatomic locations (Litwin et al., 2017), and the similarity further strengthens the causal role between HPV and subgroups of SCC arising in the conjunctiva and LDS.

TP53 is the most commonly affected gene in cancer (Mendiratta et al., 2021), encoding the "guardian of the genome" – p53. Not surprisingly, loss-of-function mutations in *TP53* were highly prevalent in our cohort of HPV-negative SCC. The few studies previously investigating the molecular profile of conjunctival SCC have also reported somatic mutations and structural variations in *TP53* and aberrant p53 expression as important features of these tumors (Ateenyi-Agaba et al., 2004; Lazo de la Vega et al., 2020). The HPV-negative carcinomas in our cohort were further characterized by frequent loss of *CDKN2A*, *KMT2D*, and *RB1*, as well as activating mutations in *PIK3CA* and *FGFR3*, concordant with a recent study by Lazo de la Vega et al. (2020). They further compared the mutational profile of conjunctival SCC with cutaneous eyelid SCC and found close similarities; both regarding affected genes and the genomic profile indicative of a UV-induced mutagenesis (Lazo de la Vega et al., 2020). Previous reports using genome-wide investigations have also revealed enrichment for C>T and CC>TT mutations, again indicating a UV-induced mutational profile (Galor et al., 2016; Ramos-Betancourt et al., 2020). The hypermutated UV-induced genomic profile with shared similarities to cutaneous SCC and previous reports of elevated PDL-1 expression in conjunctival SCC suggests a role for immunotherapy in the treatment of these tumors (Nagarajan et al., 2019; Wolkow et al., 2019).

HPV as a potential biomarker

In oropharyngeal SCC, HPV positivity is a biomarker of superior progression-free and overall survival (Ang et al., 2010; Quabius et al., 2015). To date, the prognostic role of the HPV status of conjunctival CIN and SCC is still uncertain, and results to date are diverging. Some studies report a more aggressive course of HPV-positive carcinomas with a higher AJCC T stage at diagnosis (Nagarajan et al., 2019;Ramberg et al., 2021a) and a higher risk of recurrence (Ramberg et al., 2021a), whereas other studies could not detect such differences (Hongo et al., 2022). Chauhan et al. (2012, 2018) reported p16 expression to correlate with a worse PFS, whereas the same group has previously reported HPV positivity to be a predictor of increased PFS. This inconsistency may be due to the low specificity of p16 to predict high-risk HPV infection, but it may also reflect the vulnerability and lack of power of these small studies. Future studies investigating the possible role of HPV as a biomarker in ocular adnexal SCC should restrict their analyses to biologically relevant infections. Furthermore, other established predictors of progression-free survival, including AJCC T stage at diagnosis, the status of the resection margins, and the application and choice of adjuvant therapy (McKelvie et al., 2002; Miller et al., 2014; Mirzayev et al., 2019; Siedlecki et al., 2016) should also be adjusted for in future studies regarding the prognostic role of HPV in these tumors.

Causality of HPV in ocular adnexal carcinomas

While our knowledge to date regarding HPV in carcinomas arising in the ocular sebaceous glands and the eyelids is still scarce (Ramberg & Heegaard, 2021), increasing evidence supports a causal relationship between HPV and a subset of carcinomas arising in the LDS and conjunctiva. Today, there is no reasonable doubt about the carcinogenic consequences of HPV (reviewed by Bosch et al., 2002). The carcinogenic effects of E6 and E7 from high-risk HPV genotypes are well substantiated, actually to such an extent that IARC, in their latest version, stated: "There is strong mechanistic evidence that HPV16 and HPV18 act directly to cause cancers in those tissues in which they are found" (IARC 2012). The deregulated expression of viral oncogenes within the tumor cells of both the conjunctiva and the LDS suggests an abortive, rather than a productive, life cycle and is a strong indicator of a causal relationship (Isaacson Wechsler et al., 2012; Evans et al., 2014; Griffin et al., 2019; Ramberg et al., 2021a; Ramberg et al., 2021b; Ramberg et al., 2022). The causal relationship is further strengthened by the pathognomonic HPV-induced histopathological and genetic profiles that are shared across anatomic sites as reviewed above. And lastly, the strong association between HPV and conjunctival CIN and SCC compared to control tissue (OR 8.2, 95% CI 3.7–19.2) reported in our previous meta-analysis of these patients (Ramberg et al., 2020a) indicates that HPV is not a commensal infection in immunocompetent individuals. However, there are still pieces missing. These include further investigations of the HPV prevalence heterogeneity in conjunctival SCC to increase the consistency and replication of the findings. Furthermore, there is a need for consequent comparisons of HPV-positive and HPV-negative tumors regarding clinical, histopathological, and genomic parameters for validation and further elaboration. Again, the importance of differentiating between transcriptional activity and the mere presence of HPV DNA has to be stressed. These are prerequisites for defining a potential diagnostic, prognostic, and predictive value of HPV in these tumors.

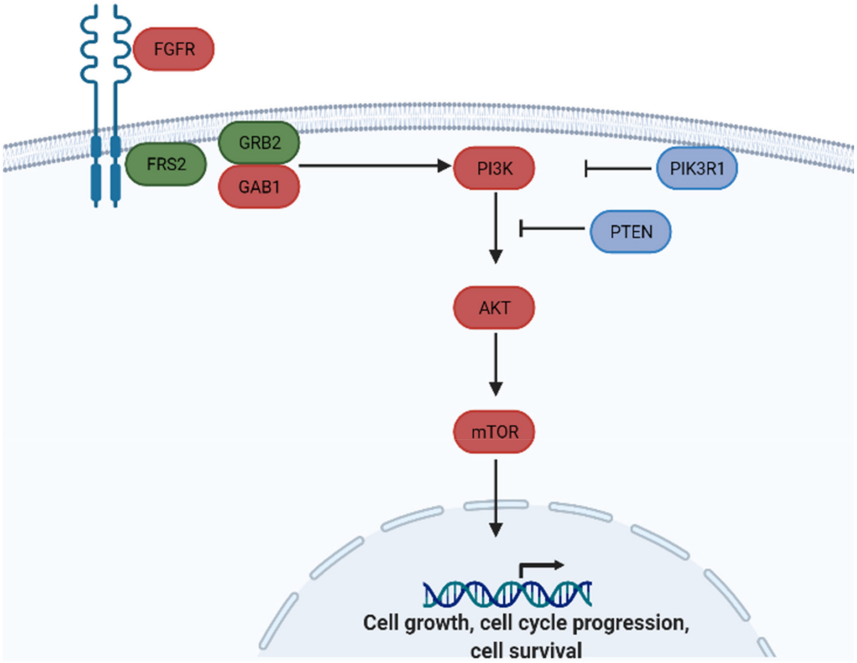

FIGURE 9 The PI3K-AKT signaling pathway (simplified). The figure is created with Biorender.com.

CONCLUSIONS

This thesis includes studies of conjunctival and LDS carcinomas, their precursor lesions, and their relation to HPV. From the present thesis, we have learned that HPV is associated with a subset of conjunctival and LDS carcinomas and their precursor lesions. We have learned that the expression of viral oncogenes is present within the tumor cells in the whole thickness of the tumor – a deregulated pattern that suggests an abortive rather than a productive infection. The high-risk HPV-positive carcinomas form a defined subset of these tumors with a distinct histological and genomic profile similar to HPV-driven carcinomas at other anatomic locations, likely driven by the PI3K-AKT signaling activation and the oncogenic effects of high-risk HPV E6/E7. Conversely, the HPV-negative counterparts harbor frequent genomic alterations in *TP53*, *CDKN2A*, and *RB1*, which were mainly unaffected in HPV-positive carcinomas at the genomic level. We have further learned that the high-risk HPV16 is the genotype associated with the vast majority of conjunctival and LDS carcinomas and that most other implicated genotypes are covered by the prophylactic HPV vaccines. The studies included in the present thesis have emphasized the conjunctival and LDS mucosa as vulnerable sites for HPV-related carcinogenesis.

FUTURE PERSPECTIVES

Squamous cell carcinoma is the most prevalent malignancy of the conjunctiva and the lacrimal drainage system, yet we have barely scratched the surface to understand the underlying molecular mechanisms. Such investigations are critical for understanding disease development and may have therapeutic implications. Topical pharmacotherapies, for example, are widely used as monotherapies and adjuvants in treating conjunctival CIN and SCC. Although a broad palette of topical pharmacotherapies is available, none of them are genetically tailored, which may explain the non-responsiveness and intolerance of the medications in, to date, undefined subgroups. Furthermore, the high recurrence rates emphasize that there are mechanisms of this diseases that we are far from understanding.

Genomic studies on conjunctival and LDS carcinomas have identified potential therapeutic candidates, including PI3K-AKT, EGFR, FGFR, and their downstream targets, which have currently available therapies. Validation and further characterization of the tumors will be enabled by high-throughput "omics" technology and translational studies. Also, reports suggest a role of immunotherapy in treating these patients, which deserves further investigations.

Supported by the present work, carcinomas of the LDS and conjunctiva have distinct phenotypes depending on HPV status, implying that the ocular adnexal mucosa is a vulnerable site for transforming HPV infections. Future research will further elaborate on the diagnostic, prognostic, and predictive value of HPV as a biomarker in these diseases. The rarity of the tumors makes large studies hardly feasible, and multicenter collaborations will therefore be of utmost importance to improve the outcome of these patients. Due to shared genomic and phenotypic characteristics of HPV-positive carcinomas across sites, HPV-positive ocular adnexal SCC may be eligible for inclusion in future basket trials. Improvements in diagnosis and treatment may also follow the extrapolation of data from trials of the corresponding tumors in the head and neck region that share viral, histopathological, and genomic features. Close attention should be paid to the clinical trials of HPV-directed therapy, which may benefit patients suffering from HPV-positive LDS carcinomas.

Today, the prevention of several types of HPV-related neoplasia is possible by HPV vaccines. Since the prophylactic HPV vaccines cover most genotypes related to ocular adnexal neoplasms, they are also expected

to prevent HPV-positive neoplasms in this region. However, such effects in vivo will take years to manifest due to the latency between infection and carcinoma development. Results from other anatomic locations suggest that prophylactic vaccines could be administrated as an adjuvant to HPV-positive intraepithelial neoplasia and papilloma to reduce the risk and time to recurrence. However, adjuvant vaccinations in transforming infections have not shown efficacy. Therefore, substantial efforts have been put into developing therapeutic vaccinations of already established HPV infections. Most therapeutic vaccines are designed to stimulate a selective cellular immune response against the HPV E6 and E7 oncoproteins instead of humoral response against HPV L1. Although no therapeutic HPV vaccines have been approved to date, several phase IIb and III studies are ongoing, in addition to great interest in specific therapies and treatment regimens of HPV-driven diseases. Future vaccine research will seek to optimize the specific cellular response and understand the tumor microenvironment for the CD8+ and regulatory T cells to reach their targets and perform their functions unrestricted. The future hope of eliminating HPV-driven disease is still alive.

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LIST OF SCIENTIFIC PAPERS

This thesis is based on the following papers, which will be referred to in the text using the following Roman numerals:

I. **Ramberg I**, Toft PB, Georgsen JB, Siersma VD, Funding M, Jensen DH, von Buchwald C, Heegaard S. Conjunctival Intraepithelial Neoplasia and Carcinoma: Distinct Clinical and Histopathological Features in Relation to Human Papillomavirus Status. *Br J Ophthalmol*. 2021. 105:878–883.

II. **Ramberg I**, Vieira FG, Toft PB, von Buchwald C, Funding M, Nielsen FC, Heegaard S. Genomic Alterations in Human Papillomavirus Positive and -Negative Conjunctival Squamous Cell Carcinoma. *IOVS*. 2021.

III. **Ramberg I**, Vieira FG, Toft PB, von Buchwald C, Heegaard S. Viral and Genomic Drivers of Squamous Cell Neoplasms Arising in the Lacrimal Drainage System. *Cancers*. 2022. 14:2558.

IV. **Ramberg I** and Heegaard S. Human Papillomavirus Related Neoplasia of the Ocular Adnexa. *Viruses*. 2021. 13:1522.

List of scientific papers *not* included in this thesis, but with relevance to the subject:

V. **Ramberg I**, Møller-Hansen M, Toft PB, Funding M, Heegaard S. Human Papillomavirus Infection Plays a Role in Conjunctival Squamous Cell Carcinoma: A Systematic Review and Meta-analysis of Observational Studies. *Acta Ophthalmol*. 2020. 99:478–488.

VI. **Ramberg I**, Toft PB, Heegaard S. Carcinomas of the Lacrimal Drainage System. *Surv Ophthalmol*. 2020. 65:691–707.

VII. **Ramberg I**, Heegaard S, Prause JU, Sjö NC, Toft PB. Squamous Cell Dysplasia and Carcinoma of the Conjunctiva. A Nationwide, Retrospective, Epidemiological Study of Danish Patients. *Acta Ophthalmol*. 2015. 93:663–6.

VIII. **Ramberg I**, Sjö NC, Bonde JH, Heegaard S. Inverted Papilloma of the Conjunctiva. *BMJ Open Ophthalmol*. 2019. 28:e000193.

IX. Fagerberg PS, **Ramberg I**, Toft PB. Combining Brachytherapy and Cryotherapy as Adjuvant Therapy for Squamous Cell Carcinoma of the Conjunctiva: Literature Review and Case Reports. *Ocul Oncol Pathol*. 2021. 7:77–84.

X. Henriksen JR, **Ramberg I**, Mikkelsen LH, Heegaard S. The Role of Infectious Agents in Cancer of the Ocular Region. *APMIS*. 2020. 128:136–149.

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APPENDIX

A1: Literature search strategies for study IV in MEDLINE via PubMed

((((HPV) OR (Human papillomavirus*)) OR (Wart)) AND ((((((Conjunctiv*) OR (Anterior eye)) OR (Ocular surface)) OR (Limbus cornea*)) OR (Cornea*)) OR (Limbal))) AND ((((((((((Carcinom*) OR (SCC)) OR (SCCC)) OR (Dysplas*)) OR (Tumour*)) OR (Cancer*)) OR (Malignan*)) OR (Epithelioma)) OR (Epithelial neoplas*)) OR (OSSN)) OR (Squamous neoplasia*)).

A2: TRUSIGHT ONCOLOGY 500 (TSO500) GENE PANEL FROM ILLUMINA

ABL1	CDKN2C	FAS	HOXB13	MRE11A	PPARG	SOX2
ABL2	CEBPA	FAT1	HRAS	MSH2	PPM1D	SOX9
ACVR1	CENPA	FBXW7	HSD3B1	MSH3	PPP2R1A	SPEN
ACVR1B	CHD2	FGF1	HSP90AA1	MSH6	PPP2R2A	SPOP
AKT1	CHD4	FGF10	ICOSLG	MST1	PPP6C	SPTA1
AKT2	CHEK1	FGF14	ID3	MST1R	PRDM1	SRC
AKT3	CHEK2	FGF19	IDH1	MTOR	PREX2	SRSF2
ALK	CIC	FGF2	IDH2	MUTYH	PRKAR1A	STAG1
ALOX12B	CREBBP	FGF23	IFNGR1	MYB	PRKCI	STAG2
ANKRD11	CRKL	FGF3	IGF1	MYC	PRKDC	STAT3
ANKRD26	CRLF2	FGF4	IGF1R	MYCL1	PRSS8	STAT4
APC	CSF1R	FGF5	IGF2	MYCN	PTCH1	STAT5A
AR	CSF3R	FGF6	IKBKE	MYD88	PTEN	STAT5B
ARAF	CSNK1A1	FGF7	IKZF1	MYOD1	PTPN11	STK11
ARFRP1	CTCF	FGF8	IL10	NAB2	PTPRD	STK40
ARID1A	CTLA4	FGF9	IL7R	NBN	PTPRS	SUFU
ARID1B	CTNNA1	FGFR1	INHA	NCOA3	PTPRT	SUZ12
ARID2	CTNNB1	FGFR2	INHBA	NCOR1	QKI	SYK
ARID5B	CUL3	FGFR3	INPP4A	NEGR1	RAB35	TAF1
ASXL1	CUX1	FGFR4	INPP4B	NF1	RAC1	TBX3
ASXL2	CXCR4	FH	INSR	NF2	RAD21	TCEB1
ATM	CYLD	FLCN	IRF2	NFE2L2	RAD50	TCF3
ATR	DAXX	FLI1	IRF4	NFKBIA	RAD51	TCF7L2
ATRX	DCUN1D1	FLT1	IRS1	NKX2-1	RAD51B	TERC
AURKA	DDR2	FLT3	IRS2	NKX3-1	RAD51C	TERT
AURKB	DDX41	FLT4	JAK1	NOTCH1	RAD51D	TET1
AXIN1	DHX15	FOXA1	JAK2	NOTCH2	RAD52	TET2
AXIN2	DICER1	FOXL2	JAK3	NOTCH3	RAD54L	TFE3
AXL	DIS3	FOXO1	JUN	NOTCH4	RAF1	TFRC
B2M	DNAJB1	FOXP1	KAT6A	NPM1	RANBP2	TGFBR1
BAP1	DNMT1	FRS2	KDM5A	NRAS	RARA	TGFBR2
BARD1	DNMT3A	FUBP1	KDM5C	NRG1	RASA1	TMEM127
BBC3	DNMT3B	FYN	KDM6A	NSD1	RB1	TMPRSS2
BCL10	DOT1L	GABRA6	KDR	NTRK1	RBM10	TNFAIP3
BCL2	E2F3	GATA1	KEAP1	NTRK2	RECQL4	TNFRSF14
BCL2L1	EED	GATA2	KEL	NTRK3	REL	TOP1
BCL2L11	EGFL7	GATA3	KIF5B	NUP93	RET	TOP2A
BCL2L2	EGFR	GATA4	KIT	NUTM1	RFWD2	TP53
BCL6	EIF1AX	GATA6	KLF4	PAK1	RHEB	TP63
BCOR	EIF4A2	GEN1	KLHL6	PAK3	RHOA	TRAF2
BCORL1	EIF4E	GID4	KMT2B	PAK7	RICTOR	TRAF7
BCR	EML4	GLI1	KMT2C	PALB2	RIT1	TSC1
BIRC3	EP300	GNA11	KMT2D	PARK2	RNF43	TSC2
BLM	EPCAM	GNA13	KRAS	PARP1	ROS1	TSHR
BMPR1A	EPHA3	GNAQ	LAMP1	PAX3	RPS6KA4	U2AF1
BRAF	EPHA5	GNAS	LATS1	PAX5	RPS6KB1	VEGFA
BRCA1	EPHA7	GPR124	LATS2	PAX7	RPS6KB2	VHL

BRCA2	EPHB1	GPS2	LMO1	PAX8	RPTOR	VTCN1
BRD4	ERBB2	GREM1	LRP1B	PBRM1	RUNX1	WISP3
BRIP1	ERBB3	GRIN2A	LYN	PDCD1	RUNX1T1	WT1
BTG1	ERBB4	GRM3	LZTR1	PDCD1LG2	RYBP	XIAP
BTK	ERCC1	GSK3B	MAGI2	PDGFRA	SDHA	XPO1
C11orf30	ERCC2	H3F3A	MALT1	PDGFRB	SDHAF2	XRCC2
CALR	ERCC3	H3F3B	MAP2K1	PDK1	SDHB	YAP1
CARD11	ERCC4	H3F3C	MAP2K2	PDPK1	SDHC	YES1
CASP8	ERCC5	HGF	MAP2K4	PGR	SDHD	ZBTB2
CBFB	ERG	HIST1H1C	MAP3K1	PHF6	SETBP1	ZBTB7A
CBL	ERRFI1	HIST1H2BD	MAP3K13	PHOX2B	SETD2	ZFHX3
CCND1	ESR1	HIST1H3A	MAP3K14	PIK3C2B	SF3B1	ZNF217
CCND2	ETS1	HIST1H3B	MAP3K4	PIK3C2G	SH2B3	ZNF703
CCND3	ETV1	HIST1H3C	MAPK1	PIK3C3	SH2D1A	ZRSR2
CCNE1	ETV4	HIST1H3D	MAPK3	PIK3CA	SHQ1	
CD274	ETV5	HIST1H3E	MAX	PIK3CB	SLIT2	
CD276	ETV6	HIST1H3F	MCL1	PIK3CD	SLX4	
CD74	EWSR1	HIST1H3G	MDC1	PIK3CG	SMAD2	
CD79A	EZH2	HIST1H3H	MDM2	PIK3R1	SMAD3	
CD79B	FAM123B	HIST1H3I	MDM4	PIK3R2	SMAD4	
CDC73	FAM175A	HIST1H3J	MED12	PIK3R3	SMARCA4	
CDH1	FAM46C	HIST2H3A	MEF2B	PIM1	SMARCB1	
CDK12	FANCA	HIST2H3C	MEN1	PLCG2	SMARCD1	
CDK4	FANCC	HIST2H3D	MET	PLK2	SMC1A	
CDK6	FANCD2	HIST3H3	MGA	PMAIP1	SMC3	
CDK8	FANCE	HLA-A	MITF	PMS1	SMO	
CDKN1A	FANCF	HLA-B	MLH1	PMS2	SNCAIP	
CDKN1B	FANCG	HLA-C	MLL	PNRC1	SOCS1	
CDKN2A	FANCI	HNF1A	MLLT3	POLD1	SOX10	
CDKN2B	FANCL	HNRNPK	MPL	POLE	SOX17	

A3: INGENUITY VARIANT ANALYSIS FILTERING CRITERIA OF VARIANT CALL FILES

Filter	Inclusion criteria
Confidence	Call quality >20
	Variant passed upstream pipeline filtering
	Read depth > 100
	Allele fraction >10
	Outside top 5% most exonically variable 100base windows in healthy public genomes
	Outside top 1% most exonically variable genes in healthy public genomes (100Genomes)
Common variants	<1% of all in gnomAD
	<1% of all in ExAc
	<1% of all in NHLBI ESP exomes
	<1% of all in the 1000 Genomes Project
Predicted deleterious	Pathogenic variants (ACMG Guidelines classification)
	Likely pathogenic variants (ACMG Guidelines classification)
	Uncertain significance (ACMG Guidelines classification)
	Variants listed in HGMD, ClinVar, CentoMD
	Variants established in the literature