ORIGINAL INVESTIGATION

SQUAMOUS CELL CARCINOMA IN THE ANOPHTHALMIC SOCKET-A SERIES OF 4 CASES
WITH HPV-16 PROFILING.

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Synopsis: Squamous Cell Carcinoma can rarely occur in the conjunctiva of anophthalmic sockets. The exact cause is unclear, but it is likely that human papillomavirus may play a role.

All authors contributed to the design and implantation of this research, to the analysis of the results and writing of the manuscript.
ABSTRACT

Purpose: To present the clinical and histological features of squamous cell carcinoma (SCC) in the anophthalmic socket in 4 adult patients, and to determine the presence of human papillomavirus infection (HPV).

Methods: Retrospective case series of 4 adult patients with SCC of the anophthalmic socket. P16 immunohistochemistry and HPV testing was carried out in all cases. The authors report clinical findings, histopathology, management and outcomes for all 4 patients with conjunctival SCC. Previously reported cases of conjunctival SCC in anophthalmic sockets were reviewed.

Results: Four adult patients presented with eyelid lumps, discharge or change in prosthesis fit. Common examination findings included papillomatous changes, eyelid masses and epithelial changes. Three out of the four cases (75%) were positive for p16 by immunohistochemistry and the same cases positive for HPV-16 DNA. All patients received cryotherapy, topical or intralesional chemotherapy. Two patients proceeded to exenteration for control of invasive disease.

Conclusions: To the authors’ knowledge, this is the largest series of SCC in the anophthalmic socket with comprehensive annotation of HPV status. Although socket conjunctiva is protected from environmental radiation, there is still a risk of neoplastic transformation in this tissue, thus patient education and regular checking of sockets by ophthalmologists should be undertaken as a preventative measure. The potential role of HPV in these tumours warrants further investigation.
Introduction

Squamous cell carcinoma (SCC) of the conjunctiva is an uncommon condition, with a worldwide incidence ranging from 0.02 to 3.5 per 100000.\textsuperscript{1} It typically presents in sun exposed ocular surface, at the limbus, in elderly Caucasian men.\textsuperscript{2} Typical presenting features include a gelatinous, velvety or papilliform growth with leukoplakic, nodular or diffuse growth at the limbus and a complaint of irritation or redness of the eye.\textsuperscript{2} Known predisposing factors include cigarette exposure, radiation exposure, eczema / atopy, systemic immune suppression, human immunodeficiency virus infection and xeroderma pigmentosum.\textsuperscript{1}

Conjunctival SCC arising in the anophthalmic socket has been reported in 16 cases in the English language literature since 1998. The majority have been presumed to be related to chronic prosthesis use and related irritation, given that actinic exposure is minimal or absent in these tissues due to prosthesis wear. However a recent report by Gaier and coworkers reported HPV-16 positivity in one case, and postulated an association with this virus.\textsuperscript{3}

Human papillomavirus is a small epitheliotropic, non-enveloped DNA virus with more than 200 genotypes identified, of which around 13 “high risk” types are associated with certain anogenital cancers and a component of oropharyngeal cancer.\textsuperscript{4} The steps in HPV-driven carcinogenesis include infection, persistence of infection, progression to premalignant lesions and invasion.\textsuperscript{5} In the absence of invasive disease, precursor lesions can be reversible. The most carcinogenic type is HPV-16 which is an order of magnitude more oncogenic than any other high risk type. While most infection follows a subclinical or benign trajectory; the non lytic nature of the virus and the comparative lack of immunological effector cells in the epithelium enables some infections to persist, which can subsequently render a malignant phenotype through the deregulated expression key oncoproteins E6 and E7.\textsuperscript{6} While the nature and characteristics of a latent period for HPV infection is less well understood compared to other viruses, there is evidence that HPV can persist in basal cells for several years before reactivation as a consequence of external (uncharacterised) stimuli.\textsuperscript{7}
Most conjunctival papillomas are associated with HPV 6 and 11 – low risk types, although reports of high risk HPV in conjunctival papillomas, dysplasias and cancers also exist. The role of high risk HPV in conjunctival squamous carcinoma remains unclear and this is consistent with outputs from the International Agency for Research on Cancer which indicate the evidence for a role of HPV is limited. There is significant evidence, however, for the increasing burden of HPV associated cancer in the United Kingdom (UK) and national registry data indicate a rise in vulval, anal, penile and vaginal cancer, all of which have an HPV component.

An awareness of risk factors can lead to increased screening and early detection in populations at risk for a disease. In addition, earlier aggressive treatment of low grade tumours may help reduce the progression to invasive disease and burden of cancer in those with high risk features. Furthermore the increasing burden of HPV-driven head and neck cancer justify an assessment of HPV status for this rare but morbid group of tumours. The aim of this study was to characterise a consecutive series of four patients presenting with conjunctival squamous cell carcinoma in the anophthalmic socket with respect to clinical, histological and virological features.

**Methods**

A review of the records from the Wolverhampton Eye Infirmary and the Ocular Oncology Service at the Royal Hallamshire Hospital for the diagnosis of conjunctival SCC was performed. Patients wearing a cosmetic prosthesis overlying the socket, presenting from 2012-2017 were included in this retrospective case series. The notes were reviewed for demographic features, medical history, presenting symptoms; surgical procedures, local treatments, and the response to treatment during follow up. The patient photography database was also reviewed. The patients gave routine consent to have the biopsies and further surgery performed. The work was done in accordance with the Declaration of Helsinki.

All surgically removed tissues were fixed in standard buffered formalin for up to 24 hours, processed to wax and 4 micron sections cut and stained with Haematoxylin and Eosin (H&E). All cases were tested with p16 immunohistochemistry staining with
p16 ready to use antibody (Roche, Brighton UK). Antigen retrieval was carried out using the Dako low pH target retrieval solution. The primary incubation was for 20 minutes, followed by a 20 minute horse radish peroxidase step, followed by a DAB 5 minute step (with a brown end point). The immunohistochemistry was carried out in the Dako OMNIS system. Nucleic acid extraction of 10 um sections of the relevant FFPE block was performed using the reagents within the DNA mini kit (Qiagen, Hilden, Germany) with a protocol adapted to maximise HPV nucleic acid recovery. DNA extracts were then tested for HPV using the Optiplex HPV Genotyping Kit (Diamex GmbH, Heidelberg, Germany) according to manufacturer’s instructions. This genotyping test detect 24 HPV types including all established high-risk types and is the assay used for national HPV surveillance in Scotland to determine impact of the HPV vaccine.

Case Series

CASE 1
A 59-year-old man was involved in a motor vehicle accident 30 years prior to presentation, causing a ruptured right globe and subsequent enucleation. He had since worn multiple prostheses, and had no specific problems with fit. He presented with a 2-year history of a lump on the right upper eyelid. There was no discharge. Examination revealed right upper eyelid swelling with a firm central mass and downward displacement of the prosthesis. Ulceration and abnormal vasculature were noted on lid eversion (Figure 1a-b). The patient was a smoker of 15 cigarettes per day, and took medication for high blood pressure and cholesterol. His work history did not reveal exposure to significant ultraviolet radiation or chemicals.

 Conjunctival map biopsies revealed in-situ squamous cell carcinoma of the superior tarsus. He had further biopsies 2 months later, which showed invasive squamous carcinoma. The disease was not controlled with cryotherapy, and he proceeded to right orbital exenteration, 3 months post presentation. Histology revealed upper lid invasive, moderately differentiated squamous cell carcinoma (Fig 2A and 2B). The invasive carcinoma was seen to arise from extensive in-situ carcinoma within the
upper tarsal conjunctiva (Fig 2C). The invasive and in-situ components expressed p16 within the nuclei and cytoplasm (Fig 2D) and HPV genotyping demonstrated the presence of HPV 16 DNA.

CASE 2
A 65 year-old female had a history of right enucleation, 63 years earlier, for treatment of retinoblastoma. She had multiple prostheses since that time, but most recently had a new prosthesis 3 months prior to presentation. She was referred from a nearby hospital with a new lump in her lower fornix and mucoid discharge from her socket. Examination revealed mild erythema of the medial upper and lower eyelids, with abnormal conjunctival vasculature noted on lower lid eversion (Figure 1c-d).

The patient had a history of treated breast carcinoma, osteoarthritis and granuloma annulare. She was a non-smoker and had no significant ultraviolet or chemical exposure.

The patient had a conjunctival biopsy prior to referral, which showed severely dysplastic squamous epithelium, with no underlying stromal tissue. The absence of the latter prevented the assessment for the presence or absence of invasive tumour. HPV genotyping and p16 status was negative. She was treated with cryotherapy and subconjunctival interferon alpha-2a. Repeat biopsies, three months post treatment showed no residual dysplasia.

CASE 3
A 63 year-old male had a left enucleation at the age of 21 for management of severe globe injury. He had lid surgery for better prosthesis fit 10 years prior to presentation. At presentation, he reported 6 months of a lump in the socket, and gradual prosthesis migration nasally. He had no significant past medical history and was a nonsmoker with no significant occupational chemical or ultraviolet exposure.
Examination revealed left ptosis, with a palpable mass of the upper eyelid. Lid eversion revealed papillary changes and abnormal vasculature. He was booked for map biopsies at presentation, which revealed papillary in-situ SCC. He was treated with intralesional interferon and cryotherapy. After cessation of therapy, he had rapid growth of the socket lesion, and proceeded to left orbital exenteration. Histology revealed an invasive moderate to poorly differentiated squamous cell carcinoma of the superior bulbar conjunctiva arising from papillary in-situ SCC (Fig 1e-f). The tumour was positive for p16 (Fig 2G) and HPV 16 DNA.

CASE 4
A 73-year-old female had an injury to her right eye at the age of 19. The eye was salvaged, but she had an enucleation 10 years prior to presentation for a blind, painful eye. She was referred with a 6 month history of a right upper eyelid lump.

This patient was a non-smoker, but suffered severe eczema, and took medication for treatment of hypertension, osteoarthritis and asthma. She did not have a history of significant environmental chemical or ultraviolet exposure.

On presentation, examination revealed a mass of the medial upper eyelid, with leukoplakia and papillomatous changes on lid eversion (Figure 1g-h). She had a biopsy at the referring hospital, which revealed papillary in-situ SCC with a prominent population of eosinophils (Fig 2H), reflecting the patient’s atopic state. The patient proceeded to intralesional interferon on the day of presentation at our unit. She had a further wedge excision of the affected eyelid, which revealed extensive in-situ squamous cell carcinoma. The tumour was positive for p16 and HPV 16 DNA. She was successfully treated with three cycles of topical mitomycin C 0.04%.

The key case findings including HPV/p16 status are summarised in Table 1

Discussion
There have been 16 cases of conjunctival SCC reported in anophthalmic sockets in the literature to date (Table 2). The current study brings the total number of
reported cases to 20. The mean age at presentation was 58 years, with a mean of 42 years of prosthesis wear. The disease is seen to occur more commonly in males (17 out of 20). The most common presenting features are a mass in the socket (50%), poor prosthesis fit (40%) and mucoid discharge (25%). Ten of the 20 reported cases (50%) arose in the tarsal or fornical conjunctiva of the upper eyelid. The majority of reported cases (55%) were treated with exenteration, although a recent study by Shields and coworkers reported a series of 3 patients all successfully treated with topical and intralesional interferon alpha-2b.¹

Squamous cell carcinoma of the palpebral conjunctiva and the adjacent lower eyelid skin presents a unique dilemma in the determination of a common aetiology. This is particularly true in the context of a prosthesis offering protection from ultraviolet exposure and other environmental irritants. Traditional hypotheses have related to the chronic friction caused by decades of prosthesis wear, leading to conjunctival leukoplakia and intraepithelial neoplasia.¹⁰ Frictional keratosis and leukoplakia associated with underlying squamous cell malignant change is well described in the oral mucosa.¹¹ In oral cancer, the role of chronic trauma of the oral mucosa contributing to carcinogenesis has been shown to be multifactorial: increased mitosis to repair tissue injury put cells at risk of DNA damage by other agents, initiating carcinogenesis, release of chemical mediators and / or oxidative stress, inhibiting DNA repair, altering transcription factors, preventing apoptosis and stimulating angiogenesis.¹¹

There is evidence for a similar mechanism of chronic trauma being relevant to the development of conjunctival tumours.¹⁰ Physical and inflammatory irritation of the conjunctival basement membrane stimulating the oncogenic potential of overlying epithelial cells has been proposed as an aetiological factor in SCC development in the presence of mucous membrane pemphigoid.¹² Poorly fitting prostheses inducing conjunctival inflammation and epithelial dysplasia with eventual transformation into carcinoma is a favoured cause for the development of conjunctival SCC in anophthalmic sockets.¹⁰,¹³-¹⁵
The hypothesis of friction from a poorly fitting prosthesis leading to the development of conjunctival SCC can be addressed by a recent histological study of conjunctiva in anophthalmic sockets. Kim and colleagues designed a study to determine if a correlation existed between particular aspects of ocular prosthesis care, including total wearing time and frequency of polishing, and cytologic features of the conjunctival epithelium in impression cytology. They found squamous metaplasia with decreased goblet cell density and increased nucleus-to-cytoplasm ratio occurred in the conjunctiva of anophthalmic sockets, however this was not associated with particular aspects of prosthesis care. Squamous metaplasia, however, is well documented to precede the development of dysplasia in other mucosal carcinomas. The upper tarsal or forniceal conjunctiva was seen to be involved in 50% of reported cases of SCC in anophthalmic sockets. This is in contrast to the majority of conjunctival SCCs which are known to occur most frequently at the limbus or bulbar conjunctiva. The upper eyelid makes the largest excursion over the prosthesis in an anophthalmic socket, rendering the tissue lining most susceptible to inflammatory change, metaplasia and microtrauma.

High-risk human papilloma virus is a well-established causative agent of a component of oropharyngeal carcinomas and cancers of the anogenital tract including cervical cancer. The association of HPV-16 and carcinomatous transformation was extended to the conjunctiva in 1989 when McDonnell and coworkers identified the virus in all patients investigated with conjunctival SCC. There has been a marked variation in detection rates of HPV in periocular SCC and precursors since that time, ranging from 0 to 100%. This variation is likely due to differing techniques to identify HPV in biological specimens and differing study designs. In 2016 Afrogeh and coauthors were the first to examine the incidence of HPV in ocular surface squamous neoplasia using an accepted standardised combination of p16 and in-situ hybridisation. Using these methods they found HPV-16 in 30% of conjunctival squamous neoplasia.

In order to determine whether HPV is a common ‘passenger’ on the ocular surface, Sjö and coworkers compared conjunctival papillomas and normal conjunctival
tissue. They found a strong association between HPV and conjunctival papilloma, with types 6 and 11 to be the most frequently detected. In addition, they found all normal conjunctival biopsy specimens to be negative for HPV.

The current study found 3 of 4 patients (75%) were positive for HPV-16 using a very sensitive PCR based assay and all three cases were also positive for p16. p16 is the product of the INK-4 gene and is a cellular cyclin dependent kinase inhibitor which under normal circumstances exerts a tumour suppressor role. Accumulation of p16 can be triggered by over expression of the E7 oncoprotein of high risk HPV and p16 is sometimes referred to as a secondary marker of HPV infection as a consequence. Given the ubiquity of HPV infection and sensitivity of PCR based testing, it is possible that HPV DNA can be detected in a malignant lesion, yet not be driving it or be present as an external contaminant. Furthermore, it is possible for p16 to be upregulated in the absence of HPV infection (eg. in some cases of metaplasia). Thus, for comprehensive annotation of HPV status of a lesion both p16 and HPV molecular testing should be performed, as a double-positive result confers more robust evidence that HPV has an active role in the lesion.

A review of the pathophysiology of ocular surface squamous neoplasia was recently published which detailed the mechanism of solar ultraviolet radiation, human immunodeficiency virus (HIV) and HPV in the transformation of limbal stem cells and the development of the disease. The presence of HPV-16 in both benign and malignant conjunctival lesions has been reported – suggesting that the virus alone is incapable of initiating squamous cell carcinoma, but may be a cofactor. Gichuhi and colleagues identified micro-abrasions of the ocular surface caused by Vitamin A deficiency as a risk factor in conjunctival SCC, allowing HPV to invade the conjunctival basement membrane and epithelial cells. We propose that similar microabrasions could be caused by long-term prosthesis wear.

Detecting SCC in the socket is significantly more challenging as the colour contrast of the white scleral background is absent, and the vascular tumour blends with the pink colour of the socket. Tumours are further camouflaged in the fornix. Typically, SCC
and its precursors show an array of hairpin loops within the mass. Despite these pitfalls for the clinician, a thorough examination of all anophthalmic patients, or those wearing prostheses should be carried out annually for early detection of abnormalities. Patients should be warned to present if they have new masses, discharge or altered prosthesis centration.

In conclusion, although exceedingly rare, SCC can develop in the anophthalmic socket. It typically presents in males in the fifth decade, with 40-45 years of prosthesis wear. Characteristic presenting complaints include a mass or altered prosthesis fit. In 3 of the 4 cases HPV-16 was detected which was consistent with the p16 result indicating oncogenic activity of HPV within these lesions. Further interrogation of larger national series of SCC in anophthalmic socket may indicate whether HPV is playing an increasing role in these cancers as has been observed for other neoplasms in the UK.
<table>
<thead>
<tr>
<th>Case number</th>
<th>Main histopathology findings</th>
<th>p16 immunohistochemical status</th>
<th>Human papillomavirus 16 status</th>
</tr>
</thead>
</table>
| 1           | a. Mapping biopsies: extensive in-situ SCC on mapping biopsies.  
              b. Further biopsy from superior tarsus-invasive SCC  
              c. Exenteration: Invasive SCC and in situ SCC. | Positive | positive |
| 2           | a. Index biopsy: Granulation tissue covered partly with in-situ SCC with foci suspicious for invasion  
              b. Post treatment biopsies: No dysplasia | negative | negative |
| 3           | a. Index biopsy: Papillomatous in situ SCC  
              b. Exenteration: invasive SCC with comedo necrosis, arising from papillary in-situ SCC. | positive | positive |
<p>| 4           | a. Index biopsy In-situ SCC with numerous eosinophils | positive | positive |</p>
<table>
<thead>
<tr>
<th>Author (year)</th>
<th>No. of Cases</th>
<th>Age / Sex</th>
<th>Prosthesis Use (yrs)</th>
<th>Presenting Features</th>
<th>Treatment</th>
<th>Location</th>
<th>Follow Up (months)</th>
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<tr>
<td>Campanella (1998)</td>
<td>2</td>
<td>69 / M</td>
<td>49</td>
<td>Serosanguineous discharge, discomfort, mass</td>
<td>Exenteration</td>
<td>Superior Tarsal Conjunctiva</td>
<td>-</td>
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<tr>
<td></td>
<td></td>
<td>77 / M</td>
<td>43</td>
<td>Discomfort, swelling</td>
<td>Exenteration</td>
<td>Superior Fornix</td>
<td>-</td>
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<tr>
<td>Whittaker (2002)</td>
<td>1</td>
<td>62 / M</td>
<td>49</td>
<td>Mass, yellow discharge</td>
<td>Excisional Biopsy</td>
<td>Lower Lid and Tarsal Conjunctiva</td>
<td>7</td>
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<tr>
<td>Endo (2006)</td>
<td>1</td>
<td>51 / M</td>
<td>40</td>
<td>Poor prosthesis fit</td>
<td>Exenteration</td>
<td>Lower conjunctiva</td>
<td>15</td>
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<tr>
<td>Chaudhry (2006)</td>
<td>1</td>
<td>50 / M</td>
<td>50</td>
<td>Irritation</td>
<td>Excisional Biopsy</td>
<td>Socket</td>
<td>36</td>
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<tr>
<td>Nguyen (2008)</td>
<td>2</td>
<td>58 / M</td>
<td>51</td>
<td>Poor prosthesis fit, sanguinous discharge</td>
<td>Exenteration</td>
<td>Upper Bulbar Conjunctiva</td>
<td>12</td>
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<tr>
<td></td>
<td></td>
<td>56 / M</td>
<td>44</td>
<td>Poor prosthesis fit</td>
<td>Exenteration</td>
<td>Bulbar conjunctiva</td>
<td>24</td>
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<td>Hsu (2009)</td>
<td>1</td>
<td>75 / M</td>
<td>63</td>
<td>Mass</td>
<td>Exenteration and XRT</td>
<td>Socket</td>
<td>24</td>
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<td>Jain (2010)</td>
<td>1</td>
<td>60 / M</td>
<td>53</td>
<td>Swelling, poor prosthesis retention</td>
<td>Exenteration</td>
<td>Upper bulbar and palpebral Conjunctiva</td>
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<td>Barrett (2010)</td>
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<td>32</td>
<td>Mucous discharge, poor prosthesis fit</td>
<td>Topical MMC</td>
<td>Superior Fornix</td>
<td>10</td>
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<tr>
<td>Espana (2011)</td>
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<td>62 / M</td>
<td>60</td>
<td>Mass</td>
<td>Excisional Biopsy, MMC, Cryo, Exenteration</td>
<td>Superior Fornix</td>
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<td>Shields (2011)</td>
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<td>60 / F</td>
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<td>Nodule</td>
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<td>Superior forniceal conjunctiva and caruncle</td>
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<td>Superior Tarsal, bulbar and Forniceal</td>
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<td>Gender</td>
<td>Diagnosis</td>
<td>Treatment</td>
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<td>Jakobiec (2016)²</td>
<td>1</td>
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<td>Mass, poor prosthesis fit</td>
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<td>Inferior Fornix</td>
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<td>Mucopurulent discharge, poor prosthesis fit</td>
<td>Exenteration, 60Gy 30# XT</td>
<td>Diffuse</td>
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<td>59</td>
<td>M</td>
<td>Mass</td>
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<td>Superior tarsal</td>
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<td></td>
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<tr>
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<td>M</td>
<td>42</td>
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<td></td>
<td>73</td>
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<td>10</td>
<td>Mass</td>
<td>Excisional biopsy, MMC</td>
<td>Superior tarsal</td>
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Figure Legends

Figure 1 (a to h):

External photograph with conjunctival appearance for patient 1 to 4.
A. Case 1 Scanning power H&E image of the upper lid invasive conjunctival squamous cell carcinoma.
B. Case 1-Higher power of A showing the invasive tumour.
C. Case 1-Higher power of A showing the in-situ squamous carcinoma.
D. Case 1: p16 immunohistochemistry (brown=positive) showing positivity in the in-situ and invasive tumour (main figure). The top-right inset figure shows a higher power of the p16 staining, demonstrating nuclear and cytoplasmic staining.

E. Case 3: Scanning power H&E image of the upper lid showing papillary in-situ squamous cell carcinoma.

F. Case 3: showing invasive squamous carcinoma infiltrating extraocular muscles (arrow).

G. Case 3: p16 immunohistochemistry showing positivity of the tumour (main figure). The top-right inset figure shows a higher power of the p16 staining, demonstrating nuclear and cytoplasmic staining.

H. Case 4: showing in-situ squamous carcinoma with intra-lesional eosinophils, reflecting the patient’s atopic status.
References


