Detecting Human Papillomavirus in Ocular Surface Diseases

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PURPOSE. Human papillomavirus (HPV) infection has been implicated as a possible inducing factor for benign and neoplastic ocular surface diseases such as pterygia and ocular-surface squamous neoplasia (OSSN). However, the wide range in HPV prevalence previously reported for both diseases adds controversy to, and highlights the limitations of, this field. The aim of this study was to determine the prevalence of HPV in pterygia and OSSN and to devise a standardized approach for detecting viral DNA in ocular tissue samples.

METHODS. DNA was extracted from a variety of specimens (n = 160), including formalin-fixed paraffin-embedded tissue shavings, fresh tissue, and cultured cells. Nested PCR for HPV with consensus and subtype-specific primers was used to detect viral DNA. Confirmatory assays, including molecular sequencing, histology, and immunohistochemistry for HPV E6 protein and p16 were also performed.

RESULTS. HPV was not detected in pterygia or normal conjunctiva. However, 6.5% (3/46) of OSSN samples were HPV-positive by PCR, sequencing, and immunohistochemistry. Positive cases were all squamous cell carcinoma of the conjunctiva (SCCC), the most severe form of OSSN, representing 12.5% (3/24) of SCCCs in our cohort. HPV-16 was the genotype identified in each case and this correlated with the presence of koilocytes and intense immunoreactivity for p16. Our study found no association between pterygia and OSSN with other oncogenic viruses, such as EBV or CMV, as they were just as prevalent in normal conjunctiva.

CONCLUSIONS. The low prevalence of HPV-16 in ocular surface disease suggests infection is not a cause but a cofactor in disease development.

Keywords: conjunctiva, epithelium, virus, pterygia, neoplasia, ocular surface

Pterygia are benign, fibrovascular, inflammatory lesions that grow centripetally from the limbus toward the cornea, obscuring vision via a number of mechanisms.1 There is strong epidemiological evidence supporting chronic ultraviolet radiation (UVR) as the main triggering agent;1 however, genetic inheritance2 and oncogenic viruses3 also may play a role. The prevalence of pterygia varies depending on geographical location; it can be as high as 23% in equatorial regions4 or less than 0.7% at higher latitudes.5 Pterygia are characterized by hyperplasia, cell proliferation, and angiogenesis, and although locally invasive, they can recur and display neoplastic transformation.6,7 Modulated expression of effector molecules, such as cytokines, growth factors, and matrix metalloproteinases play an essential role in the processes that result in pterygium genesis.8–10

Ocular surface squamous neoplasia (OSSN) is the term given to a spectrum of less common lesions that range from mild to severe dysplasia, to carcinoma in situ, to squamous cell carcinoma of the conjunctiva (SCCC).11 OSSN is the most common ocular surface tumor with a worldwide incidence of 0.02 to 3.5 per 100,000.12 The most severe form, SCC, causes severe ocular morbidity, can metastasize to lymph nodes,13 but rarely results in mortality. The pathogenesis of OSSN is also poorly understood, with molecular events leading to its development yet to be fully elucidated. To date, few studies have been performed to identify candidate molecules that may be involved in disease progression.14–16 OSSN most commonly arises from the limbus15 and, like pterygia, is thought to develop from excessive UVR exposure17 or viral infection.12,18–20

In pterygia, investigators found a significant association with HPV: one study detected the virus in 100% of cases,21 others reported a prevalence of 28%,22 and some failed to detect it at all.23–25 Similarly for OSSN, researchers noted HPV in 100% of cases,26,27 whereas others were unable to detect the virus.28,29 Dushku and colleagues30 assessed both pterygia (n = 13) and limbal tumors (n = 10), but HPV was not found in any specimen. Hence, the role of HPV in both diseases remains controversial and unresolved.12

HPV is a small epitheliotropic, nonenveloped DNA virus with more than 200 genotypes recognized, but not all are fully characterized.31 The virus can be transmitted through sexual
contact, autoinoculation, or maternally. HPV-16 is regarded as one of the most carcinogenic agents known, although other high-risk viral subtypes vary in oncogenic potential. High-risk HPVs are the main cause of cervical cancer and are thought to play a role in the development of vulval, vaginal, penile, anal, and skin cancer. HPVs are the main cause of cervical cancer and are thought to play a role in the development of vulval, vaginal, penile, anal, and skin cancer. HPVs are the main cause of cervical cancer and are thought to play a role in the development of vulval, vaginal, penile, anal, and skin cancer.

HPV gains access to the epithelium through microabrasions and targets basal epithelial stem cells where strategically it can remain long-lived. There is evidence that 26-integrin and laminin 5 act as receptors for the virus. Interestingly, 26-integrin is a marker for corneolimbal epithelial stem cells. On entering the cell, in cases associated with transformation, the HPV DNA is often incorporated into the human genome. This leads to overexpression of the viral oncogenes E6 and E7, which interfere with the cell's regulation of proliferation through the degradation of p53 and Rb/p105 proteins and activation of the cyclin-dependent kinase protein p16. Interestingly, HPV-16 E6 and E7 can cause aberrant mobilization and proliferation of stem cells from an otherwise quiescent repository. These mechanisms are used by the virus to evade immune detection, ensure long-term infection, and promote immortalization and tumorigenesis.

We postulated that HPV can be detected and plays a role in the pathogenesis of prevalent ocular surface diseases. To test this hypothesis, we assessed fresh and formalin-fixed archival tissue and cell lines. We demonstrated that although HPV is not associated with pterygia, a significant proportion of SCCC harbor HPV-16 DNA.

**Materials and Methods**

**Tissue Specimens**

Fresh pterygia and OSSN tissue were surgically excised from patients admitted to the Prince of Wales Hospital, Sydney, Australia. Specimens were placed in sterile saline and transported to the laboratory on ice before being processed. A database search spanning December 1996 to December 2012 was conducted for formalin-fixed paraffin-embedded (FFPE) OSSN specimens. In addition, cervical cancer specimens were retrospectively collected from archival tissue blocks stored in the same hospital department (Anatomical Pathology, Prince of Wales Hospital). FFPE pterygia were obtained from our tissue repository. Hematoxylin and eosin (H&E)-stained sections of all specimens were microscopically reviewed and excluded from the study if they were deemed to have insufficient tissue for DNA extraction. Fresh control conjunctiva was harvested from human corneoscleral rims that had their central buttons excised for transplantation (Lion's NSW Eye Bank, Sydney, Australia) and were prescreened and found to be negative for hepatitis B, hepatitis C, and HIV-1 and -2. Tissues were collected in accordance with the World Medical Association Declaration of Helsinki and approved by the University of New South Wales and the Prince of Wales Human Research Ethics Committee (HREC 09/192).

**Cell Culture**

Normal human primary limbal epithelial cells were cultured in ClickTM50 media (CellnTec, Bern, Switzerland) as previously described. Pterygia and OSSN cells were revived from frozen stocks and propagated as previously detailed. In brief, selected pterygia or OSSN specimens were bisected; one segment was formalin-fixed and sent for histopathological assessment to confirm clinical diagnosis, and the other was cut into smaller pieces (1 mm³), placed into six-well culture plates as explants, covered in media, and incubated at 37°C as previously outlined. Explants were removed after 10 days, which was sufficient time for significant epithelial outgrowth without fibroblast contamination. Epithelial content in these cultures was confirmed in subcultured cells by flow cytometry using a pan cytokeratin marker (>95% purity) and p63, which indicated these cells were actively proliferating and could be propagated for 15 to 20 generations. The human cervical cancer cell lines, SiHa and HeLa, also were cultured and used as positive controls, as they carry HPV-16 and HPV-18 DNA, respectively. Cell lines were maintained at 37°C in a humidified 5% CO₂ incubator in 10% fetal bovine serum/MEM with antibiotic supplements.

**DNA Extraction From Fresh and FFPE Tissue**

FFPE tissues were acquired; ten 5-μm sections were removed from each block and placed in sterile microfuge tubes. The microtome blade was changed and equipment wiped with xylene and ethanol between blocks to avoid DNA cross-contamination. Tissue sections were de-waxed in xylene, centrifuged at 14,000g for 2 minutes, and washed in 100% ethanol thrice. Normal bulbar conjunctiva was procured from fresh corneas. Primary human ocular surface epithelial cells and human cervical cancer cell lines were collected from their plastic substratum by enzymatic dislodgment (TrypLE; Invitrogen, Eugene, OR). DNA was extracted from all specimens (GenElute Mammalian DNA Extraction Kit; Sigma-Aldrich, Sydney, Australia) and concentration measured using the NanoDrop ND1000 (Thermo Fisher Scientific, Sydney, Australia).

**PCR and Gel Electrophoresis**

All DNA samples were initially screened for the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Samples in which GAPDH was not detected (3/160) were excluded from the final analysis. In brief, 1 to 2 μL of DNA was mixed with 2 μL PCR buffer, 0.6 μL 50 mM MgCl₂, 0.4 μL 10 mM dNTPs, 0.16 μL of 1 Unit Taq Polymerase, 1 μL of each forward and reverse primer (5 μM), and nuclease-free water to a final volume of 20 μL. Thermocycling was performed using the GeneAmp PCR System 9700 (Applied Biosystems, Melbourne, Australia) and the conditions stipulated in Table 1. Specimens that returned a positive PCR product for GAPDH were screened for HPV content using nested PCR with consensus primers. The first-round PCR with MY09/MY11 primers amplified a 451-bp fragment of the L1 region common to many subtypes including, but not limited to HPV-6, -11, -16, -18, -31, and -33. The second-round PCR was spiked with 1 μL of template from the first reaction. The second reaction, with GP05/GP06 primers, amplified a 150-bp region within the product generated from the first round. Samples that were HPV⁺ based on product size were rescreened at least three times for confirmation. Samples that were HPV⁺ with consensus primers underwent further investigations using HPV subtype-specific primers (Table 1). The primer pair pU-1M/pU-2R detects HPV-16, -18, -31, -33, -52b, and -58 and generates products ranging from 251 to 268 bp. The pU-31B/pU-2R primers detect HPV-6 and -11, yielding a 228-bp product, and HPV-18-specific primers generated a PCR product of 134 bp. In addition, a multiplex PCR for detecting common pathogenic viruses (cytomegalovirus [CMV] and Epstein-Barr virus [EBV]) was performed on the same DNA. All PCR products were electrophoretically separated on agarose gels, visualized using either SYBR Safe (Life Technol-
Table 1. Primer Pairs for HPV and GAPDH PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Base Pair Position (Size)</th>
<th>Region</th>
<th>Nucleotide Sequence 5'–3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV L1 consensus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Outer PCR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MY09</td>
<td>6722–6742 (450)</td>
<td>L1</td>
<td>GC(A/C)CAGGG(A/T)CATAA(T/C)AATTGG</td>
</tr>
<tr>
<td>MY11</td>
<td>7170–7152</td>
<td>L1</td>
<td>GC(TCC(A/C)A(A/G)(A/G)GGA(A/T)ACTG</td>
</tr>
<tr>
<td>Inner PCR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GP05</td>
<td>6764–6783 (159)</td>
<td>L1</td>
<td>TTTGTTACTGTGTTGATACACTAC</td>
</tr>
<tr>
<td>GP06</td>
<td>6904–6885</td>
<td>L1</td>
<td>GAAATAAACTGTTAATTCGA</td>
</tr>
<tr>
<td>HPV-6/HPV-11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pU31B</td>
<td>400–420 (228)</td>
<td>E6–E7</td>
<td>TGCTAATTCTGGCTACCTG</td>
</tr>
<tr>
<td>pU2R</td>
<td>627–618</td>
<td>E6–E7</td>
<td>GAGCTGTGCTTAAATTGCTC</td>
</tr>
<tr>
<td>HPV-16/HPV-18</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pU1M</td>
<td>418–437 (231–268)</td>
<td>E6–E7</td>
<td>TGTCAAAAATCGTGTGTC</td>
</tr>
<tr>
<td>pU2R</td>
<td>627–618</td>
<td>E6–E7</td>
<td>GAGCTGTGCTTAAATTGCTC</td>
</tr>
<tr>
<td>HPV-18</td>
<td></td>
<td></td>
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<tr>
<td>HPV-18 L1_F</td>
<td>6599–6620 (134)</td>
<td>L1</td>
<td>TTTGTTACTGTGTTGATACAC</td>
</tr>
<tr>
<td>HPV-18 L1_R</td>
<td>6735–6709</td>
<td>L1</td>
<td>TGCAAAATCATATTTCTCACAATGTC</td>
</tr>
<tr>
<td>GAPDH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>700–719 (451)</td>
<td></td>
<td>ACCACAGTCCATGCCCATCAC</td>
</tr>
<tr>
<td>Reverse</td>
<td>1151–1132</td>
<td></td>
<td>TCCACACACCCTGTTGCTGTA</td>
</tr>
</tbody>
</table>

PCR cycling and conditions for each primer pair were based on previously published articles. 46–47 46MY09/11: Hot start 95°C 2 minutes, 40 cycles (95°C 30 seconds, 55°C 30 seconds, 72°C 30 seconds), extension 72°C 5 minutes, hold at 4°C. 46GP05/06: Hot start 95°C 2 minutes, 40 cycles (95°C 30 seconds, 55°C 30 seconds, 72°C 30 seconds), extension 72°C 5 minutes, hold at 4°C. 47pU-31B/pU-2R: Hot start 95°C 2 minutes, 40 cycles (95°C 30 seconds, 55°C 30 seconds, 72°C 30 seconds), extension 72°C 2 minutes, hold at 4°C. 47pU-1M/pU-2R: Hot start 95°C 2 minutes, 40 cycles (95°C 30 seconds, 55°C 30 seconds, 72°C 30 seconds), extension 72°C 2 minutes, hold at 4°C. HPV-18_F/HPV-18_R: Hot start 95°C 2 minutes, 40 cycles (95°C 30 seconds, 50°C 30 seconds, 72°C 30 minutes), extension 72°C 2 minutes, hold at 4°C. GAPDH_F/GAPDH_R: Hot start 95°C 2 minutes, 40 cycles (95°C 30 seconds, 60°C 30 seconds, 72°C 30 seconds), extension 72°C 2 minutes, hold at 4°C.

Sequencing

Amplicons were treated with EXOSAP-IT (USB; Affymetrix, Santa Clara, CA) at 37°C for 30 minutes to remove excess primers and dNTPs, then incubated for a further 15 minutes at 80°C to inactivate this reagent. DNA template was dispensed in two separate tubes, each with 10 pmol of forward or reverse primer (GP05/GP06), 1 μL of Big Dye Terminator (Life Technologies), and 2 μL of 5X sequencing buffer. Thermocycling was set for 26 cycles at 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes. Reaction products were precipitated with ethanol and sequenced (Ramaciotti Centre for Gene Function Analysis, University of New South Wales).

Immunohistochemistry

FFPE ocular tissues were cut (4 μm) and processed for immunohistochemistry as previously described.8–10,16,45 In brief, antigenic sites were unmasked by microwaveing sections in 0.01 M citrate buffer (pH 6.0). A mouse monoclonal antibody to HPV-16/18 E6 protein (Clone C1P5; Abcam, Cambridge, UK) was diluted (1:50) and incubated with sections overnight at 4°C. Slides were washed in Tris-buffered saline before adding a biotinylated secondary goat-anti-mouse antibody for 30 minutes. Sections were washed and incubated with horseradish peroxidase-conjugated streptavidin (Dako, Carpinteria, CA) for 1 hour and immunoreactivity developed after adding 3-amin-9-ethyl carbazole (Sigma-Aldrich). Control reactions included adding an appropriate isotype control antibody (IgG1; Dako) or deleting the primary antibody. Sections were counterstained with hematoxylin, viewed on a BX51 light microscope (Olympus, Center Valley, PA) and photographed. Immunostaining for p16 was performed using the CINtec Histology Kit (mtm Laboratories, Heidelberg, Germany) with the aid of a mouse anti-human monoclonal antibody to p16INK4a. Staining was performed on a Leica Bond III autostainer using the Bond Polymer Refine Detection Kit (Cat No. D9800; Leica, Wetzlar, Germany), which incorporates the brown chromogen 3,3′-diaminobenzidine.

Results

HPV in Ocular Biospecimens: Sensitivity and Specificity of PCR Using Consensus Primers

A total of 160 specimens, including pterygia, OSSN, normal conjunctiva, and cells derived from each of these tissues, were assessed for HPV status (Table 2, Supplementary Table S1). A representative nested PCR for HPV was performed on a sample subset. In this screen, only one OSSN sample returned a positive result (Fig. 1, upper panel, lane 11). The size of the amplicon was anticipated for HPV and corresponded to the product formed when DNA from SiHa and HeLa cells (which contain HPV-16 and HPV-18, respectively) was assessed. GAPDH was not detected in one FFPE pterygium (Fig. 1, lower panel, lane 8) and two FFPE OSSN samples (not shown), so these specimens were excluded from the final analysis.

Given that the prevalence of HPV in both pterygia and OSSN ranges between undetectable to 100%,12 it was important to gauge the sensitivity of our HPV PCR screening assay, particularly for FFPE tissues, as DNA quality can deteriorate with time.45 Using the HPV OSSN sample displayed in Figure 1 (upper panel, lane 11), a 10-fold serial dilution of the stock DNA (6 μg/mL) was used to spike each
reaction. A PCR product corresponding to HPV was generated with as little as 60 pg of DNA (Fig. 2A). Therefore, the minimum quantity of DNA required to detect HPV in these specimens was conservatively set to 600 pg. To further confirm the specificity of the PCR, five FFPE cervical cancer specimens were randomly selected from the tissue archives without prior knowledge of their HPV status. On screening their DNA, 60% (3/5) were HPV⁺ (Fig. 2B), fitting well with the global prevalence (~70%) of HPV in cervical cancer.52

After screening all specimens with nested PCR, HPV was not observed in any pterygium, normal conjunctiva, or ocular cell line. However, it was detected in three OSSN specimens, representing a prevalence of 6.5% (3/46). On closer inspection, all three specimens were from males with histopathologically confirmed SCCS, representing a prevalence of 12.5% (3/24; Fig. 2C).

Determining HPV Genotypes in SCCS

Next, type-specific primers were used to determine whether the three HPV⁺ SCCS cases were infected with low- or high-risk viral subtypes. When the pU-1M and pU-2R primers were used, two of the three samples yielded a PCR product similar in size to bands generated with DNA from HeLa and SiHa (Fig. 3A). Notably, no visible product formed when DNA from patient 2 was used, indicating that either this primer pair is less sensitive at detecting HPV or is less efficient at generating a product in the E6–E7 region of the viral genome (Fig. 3A). When HPV-18 type-specific primers were used, weak bands were detected in each sample, which matched the intensity of the product from SiHa cells that contain HPV-16 (Fig. 3B). Reassuringly, however, these bands were not as intense as those that formed when DNA from HeLa cells was used. Finally, no product was amplified when HPV-6/11-specific primers were used (Fig. 3C), indicating low-risk HPV types 6 and 11 may be absent. Reassuringly, a PCR product for the housekeeping gene GAPDH was amplified from each specimen (Fig. 3D). Each of the three HPV⁺ SCCS cases were sequenced and shown to contain HPV-16 (Supplementary Fig. S1).

Immunolocalization of HPV E6 Protein in SCCS

To corroborate the PCR and sequencing results, each HPV⁺ SCCS specimen was immunohistochemically assessed using an antibody to HPV-16/18 E6 protein. The results show specific immunoreactivity for E6 in tumor cells but not in resident stromal cells (Figs. 4A–C), fitting with HPV’s epithelial tropism.58 HeLa cells, which acted as a positive control, also displayed immunoreactivity for the E6 (Fig. 4C, inset). Sections incubated with an appropriate isotype control antibody developed no signal (Figs. 4D, 4F). In one case that had a significant inflammatory cell infiltrate, a small proportion (~1%) of plasma cells displayed intense immunoreactivity for E6 (Fig. 4B, inset). Pterygium and conjunctival tissue were stained in parallel, but no signal developed in these specimens (data not shown).

Next, we searched for additional features that could indicate viral infection in our three HPV-16⁺ SCCS. Given that high levels of the tumor suppressor protein p16 is considered a reliable marker of HPV-induced cervical cancer,27 we enquired whether this marker was present in ocular cancers. Indeed, p16 was abundantly expressed in tumor cells in each HPV-16⁺ SCCS (Figs. 5A–C), whereas HPV⁻ SCCS (Fig. 5D) and HPV⁻ pterygia (Fig. 5E) displayed minimal to no reactivity for the same protein. Another feature of HPV infection is the appearance of koliocytes.57,59 These cells were found among tumor cells in each HPV-16⁺ SCCS sample and were distinguishable by their acentric-wrinkled hyperchromatic nucleus and enlarged vacuolated cytoplasm (Figs. 5G–I).

Finally, all our DNA specimens were screened for two common pathogenic viruses by using a multiplex PCR.48 CMV was detected in 10.1% (7/69), 2.4% (1/42), and 0.0% (0/46), and EBV in 1.5% (2/69), 2.4% (1/42), and 2.2% (1/46) of normal conjunctiva, pterygia, and OSSN, respectively (Supplementary Fig. S2). Notably, these viruses were not found in the HPV-16⁺ SCCS cases.

DISCUSSION

The current study found no evidence of HPV in pterygium, but a weak association (6.5%) with OSSN; specifically we identified HPV-16 in 12.5% of SCCS. Moreover, HPV-16 correlated with viral E6 and host-derived p16 expression, as well as the development of koilocytosis. The low prevalence in our series suggests HPV infection is not the cause of either disease; rather other factors are contributors. By far the most widely accepted theory is that which proposes excessive UV radiation as the triggering agent.1,4,7,9,10,14,17 However, coinfection with oncogenic viruses, including EBV, CMV, HIV, and herpes simplex virus, can increase the risk of developing OSSN18,19,51 and pterygium.52,53 although some of these viruses were just as common in our Eye Bank control conjunctiva as in the diseased specimens we assessed (Supplementary Fig. S2).

From a recent survey of the literature, we noted the prevalence of HPV in ocular surface disease varied dramatically.12 This wide range (0%–100%) may be attributed to variations in the assays used to detect the virus, as well as geography and genetic susceptibility. From this survey, 11 of 18 studies identified HPV in pterygia, with an overall prevalence of 19%, and 9 of 16 articles detected the virus in OSSN, with an overall prevalence of 34%, representing a 2-fold increase over pterygia.12 Of note, racial composition was rarely reported. One study that incorporated specimens from two separate geographic regions found 100% of Italian patients with pterygia and 21% of Ecuadorian patients with the same disease had evidence of HPV infection,21 suggesting a geographic component. Contrary to these findings, low prevalence of HPV-containing pterygia was reported in both temperate54 and tropical55 regions, implying geography does not play a major role in susceptibility to infection but environmental and life style factors may. Another confounding factor that is not typically addressed is HIV status; this is particularly relevant because infection can increase the risk of developing OSSN by 8- to 10-fold, likely via host immunosuppression.18,19,50

Due to the myriad of methodologies that have been used, as well as differences in sensitivity between techniques, small sample sizes quoted,20,27,50 and prospective versus retrospective study designs, interpreting results from the literature has
Although PCR with consensus primers is the most widely implemented and preferred technique, the use of subtype-specific primers as the only screening test can potentially generate misleading (false-negative) results. To our knowledge, only one another study used DNA sequencing to confirm HPV in their pterygia and OSSN.

Given this is the definitive technique to confirm viral subtype and its oncogenic potential, we recommend this assay is performed whenever possible. Furthermore, appropriate reagent and specimen controls are often omitted and precautionary measures to minimize cross-contamination between specimens are rarely enforced. We addressed these issues by including appropriate cell and tissue controls, and ensuring our equipment was properly decontaminated between samples. Finally, validating results of primary screens with confirmatory assays is not typically preformed.

Notably, however, when using the same biomaterial, disparate results have been recorded, especially with PCR and less-sensitive hybridization techniques.

Our study is one of the largest to have investigated HPV infection in pterygia and the first of its kind in Australia. It is also one of the largest on OSSN and only the second to have screened for HPV in an Australian population. The other study,
**Figure 3.** HPV type-specific PCR. DNA from each HPV\(^+\) SCCC case (patients 1–3) was used as the template for reactions that contained HPV subtype-specific primers (A–C) or GAPDH (D). DNA from HeLa and SiHa cells was used as a positive control. Negative controls included reactions with primers and no template (C\(^1\)), and reactions without primers or template (C\(^2\)). A 100-bp DNA hyperladder (L) was run in an adjacent lane.

**Figure 4.** Immunolocalization of HPV-16/18 E6 in SCCC. Immunohistochemistry was used to localize E6 protein (red staining) in three HPV\(^+\) SCCC specimens (A–C). The dashed box in (B) and (E) is magnified (inset) and shows an E6-positive plasma cell (B). Inset in (C) represents HeLa cells stained with the same antibody. (D–F) Images derived from adjacent sections incubated with an isotype control antibody. All sections were counterstained with hematoxylin (blue) and photographed under oil immersion (×1000 final magnification).
conducted in Melbourne, found HPV infection in 39% of OSSN, notably higher than the prevalence recorded in the current investigation. We developed a stringent protocol to address many of the above-mentioned deficiencies. A series of assays were performed in a manner that corroborated the previous test. We recommend the initial approach incorporate a sensitive two-round nested PCR with consensus primers. Notably, several studies used a single-round PCR, which may have been insensitive to low viral copy number. We also suggest the screening assay be performed in parallel with reactions that amplify a housekeeping gene (GAPDH or \( \beta \) globin) of similar size to ensure DNA is not compromised and amenable to PCR.

Unavailability of fresh specimens is a common roadblock in these studies as tissue is typically submitted for histopathological examination. Approximately 50% of the DNA samples we enquired were derived from formalin-fixed paraffin-embedded sections. For this reason, it was decided to check the sensitivity of our PCR (Fig. 2A) and compare the results with DNA extracted from cervical cancers (Fig. 2B) and cell lines known to harbor high-risk HPVs. Furthermore, given that the nested PCR does not discriminate HPV species, we attempted to narrow its identity with primers that discriminate variants. These investigations confirmed the presence of high-risk HPVs in our cases (Figs. 3A, 3B). Interestingly, low-risk HPV-6/-11 were not detected (Fig. 3C) but were found by others. Finally, the PCR product was successfully sequenced and HPV-16 confirmed in each SCC (Supplementary Fig. S1).

**Figure 5.** Localization of p16 and koilocytes. Immunohistochemistry was used to localize p16 in three HPV-16+ SCCC specimens (A–C). Control samples included a representative HPV− SCCC (D), an HPV+ pterygium (E), and an HPV+ cervical cancer (F) specimen. Immunoreactivity is denoted by the brown staining, and hematoxylin (blue) was used as the nuclear counterstain. H&E-stained sections of the three HPV-16+ SCCC specimens (G–I) and an HPV+ cervical cancer case (J) were used to identify koilocytes (arrows). Tissue was obtained from patient 1 (A, G), patient 2 (B, H), patient 3 (C, I). Original magnification ×200 (A–F) and ×1000 oil immersion (G–J).
As well as molecular assays to confirm genomic integration of HPV, we recommend the use of histological tests, which are as not as sensitive, but ideal for identifying affected cells. Immunohistochemistry is one technique we used to localize viral E6 protein in each HPV-16+ SCCCs (Figs. 4A–C). Immunodetection in tumor, but not stromal cells, as well as in HeLa cells, confirmed specificity as an epitheliotropic virus.38,39 Furthermore, the intense staining in a minority of HeLa cells, confirmed specificity as an epitheliotropic virus.38,39 Knowledge that the tumor suppressor p16 is overexpressed in cervical cancer62 also inspired us to search for this protein. Our results demonstrated a strong correlation between intense p16 staining and HPV-16 detection (Figs. 5A–F). Finally, it has been documented that E5 and E6 viral proteins from both low- and high-risk HPVs cooperate to induce koilocytosis,50 albeit via an unknown mechanism. These unusual cells have been documented in benign conjunctival papillomas,57,63 rarely described in OSSN,63,64 but not reported in pterygia. Identification of these cells in each of the HPV-16+ SCCCs (Figs. 5G–I) suggested their presence is a reliable indicator of viral infection.

HPV-16 integration status has been linked to increased severity of cervical intraepithelial neoplasia and cervical carcinoma,65 thus rendering it tempting for us to speculate that HPV-16 infection is associated with severity of ocular surface disease, because from the spectrum of samples assessed, only SCCCs (albeit 12.5%) displayed viral integration. Overall however, our data do not support the notion that HPV is the actual cause of ocular surface disease. Furthermore, we cannot dismiss the possibility that HPV infection occurred after tumorigenesis. This is exemplified in studies of mammary gland carcinoma in which 10% (6/60) of breast cancer specimens carried HPV-16 DNA, whereas 11.6% (7/60) harbored HPV in non-neoplastic tissue.66 In addition, HPV-18 DNA was detected in 10% of pterygia, as well as in the same proportion of normal conjunctiva.22 Similarly, HPV-16 DNA was detected bilaterally in patients with unilateral OSSN,67 and patients with HPV-16+ cervical dysplasia who returned an HPV-16+ conjunctival swab had a normal ocular surface.20 These results provide no evidence to support a cause-and-effect relationship between HPV infection and neoplastic transformation, but could imply the virus remains latent in normal tissues until it encounters a trigger to promote activation.

In the future, it will be interesting to gauge the impact of the HPV vaccination program at reducing in the incidence of OSSN, particularly SCCCs. Interestingly, each of our three HPV-16+ SCCCs were male, further adding to the urgency in establishing a worldwide vaccination program for both sexes.86 The Australian government has led the way in this initiative and, as of February 2013, males aged 12 to 13 years could enroll in a school-based vaccination program (http://hpv.health.gov.au). Finally, if clinicians are alerted to the presence of high-risk HPVs, patients with ocular surface neoplasia could be carefully monitored and potentially managed with additional treatment strategies that include local antiviral/antitumoral agents, such as IFN-α/β.69

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