Human Papillomavirus DNA in Tissues and Ocular Surface Swabs of Patients with Conjunctival Epithelial Neoplasia

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DNA from human papillomavirus (HPV) type 16 has been recently identified in conjunctival epithelial dysplasia and carcinoma. In other body sites, HPV 16 is thought to play a role in the development of dysplastic lesions. To further explore the relationship between HPV and conjunctival neoplasia, we examined paraffin-embedded tissue samples from 42 biopsies or excisions from 38 patients whose lesions ranged from mild dysplasia to infiltrating squamous carcinoma of the conjunctiva. We also examined limbal swabs from six patients with dysplasia or carcinoma, five of whom also had tissue samples available for study. HPV 16 DNA was present in 37 (88.1%) tissue samples, including duplicate samples from four patients. Five (83.3%) of six patients who had conjunctival swabs had HPV 16 DNA present in the swabs, including two patients whose lesions had been excised one and eight years before swabs were done. We conclude there is a high prevalence of HPV 16 DNA in conjunctival epithelial neoplasia, suggesting that the development of neoplasia is related somehow to the presence of this virus. However, based on its presence in clinically uninvolved eyes and on the persistence of infection many years after successful eradication of the lesions, HPV apparently does not act alone in the development of conjunctival epithelial neoplasia. Invest Ophthalmol Vis Sci 33:184–189, 1992
approved by the IRB of the USC School of Medicine. Clinical information was abstracted from pathology forms submitted with tissue samples or from the patients' charts.

**Limbal Swabs**

After applying a drop of topical anesthetic, a sterile cotton swab was passed over the inferior limbus and fornix of one eye. The swab was then placed in a labeled vial containing sterile cell-free virus transport medium; a second swab and vial of medium were used for the fellow eye. Samples were stored at 4°C until analysis.

**Sample Preparation**

Paraffin-embedded tissues were cut at 6 μm, 1–4 cuts per sample. Before sectioning, knives were cleaned with 95% alcohol. The technician's gloves were changed between blocks. Water baths and paint brushes, possible sources of contamination, were not used. Sections were placed in microcentrifuge tubes and deparaffinized. For patients from whom multiple blocks were available, sections were taken from each block and placed in separate tubes. An additional section was cut from each block and stained with hematoxylin and eosin. The section was used to histopathologically determine the grade of each lesion and note the presence or absence of underlying elastotic or basalcell degeneration indicative of solar damage. Swabs were prepared for PCR by boiling, and an aliquot from each was placed in a separate tube.

**Polymerase Chain Reaction (PCR)**

The PCR was carried out as previously described9 in a mixture containing 100 μm/L of each deoxyribonucleotide, 10 μmol of Tris buffer (pH, 8.3), 50 μmol/L of potassium chloride, 1.5 μmol of magnesium chloride, and 5 μg gelatin per tube as stabilizer. Each reaction mixture also contained a pair of oligonucleotide primers specific for HPV type 16 or 18,11 at a concentration of 1 μmol/L, and 2 units of Thermus Aquaticus (Taq) polymerase (Perkin Elmer, Emeryville, California). The reaction mixture was overlaid with an equal volume of mineral oil. Temperature cycling consisted of denaturation at 94°C, annealing of oligonucleotides at 50°C for 2 minutes, and primer extension at 72°C for one minute. Thirty-five cycles were performed for HPV 16 and 40 cycles for HPV 18. Aliquots of the aqueous phase were analyzed by dot blot hybridization.

For the hybridization of amplified DNA, 5 μL of aqueous phase product was mixed with 250 μL of a denaturation solution of 0.4 N sodium hydroxide and 25 mM ethylenediaminetetraacetic acid. The mixture was applied to nylon filter membranes (Oncor, Gaithersburg MD) with a dot blot apparatus (Oncor). Samples were washed, and the DNA was fixed by baking at 80°C under 16 cm of water vacuum for 2 hours. Hybridization with 32P labeled probes specific for HPV types 16 or 18 was performed as described elsewhere.11

Positive controls included commercially available whole-genome DNA from HPV types 16 and 18 (Oncor), and conjunctival tissue previously positive for HPV DNA.9 Negative controls included paraffin sections from six pterygia, one conjunctival melanoma, and three samples of ligneous conjunctivitis. Additional negative controls included two vials run through the PCR reaction, dot blot, and hybridization containing all elements except target DNA or taq polymerase, respectively. These elements were replaced with an equal volume of distilled water.

**Results**

Forty-two tissue specimens from 38 patients were obtained. The 33 patients whose ages were known ranged in age from 22 to 88 years, with a median of 59.4 years. Nine (23.7%) of the 38 patients were women. Race was specified for 21 (55.3%) patients, 9 (42.8%) of whom were white. Twelve others (57.1%) had Latin surnames. All 38 patients had unilateral bulbar conjunctival epithelial neoplasia. One (2.6%) patient had acquired immune deficiency syndrome. None of the others had any known immunodeficiencies, although one patient had diabetes mellitus. Twelve (28.5%) samples were available as multiple tissue blocks, and tissue from more than one procedure was available from four (10.5%) patients, one of whom had a 7-year interval between surgeries.

On light microscopic evaluation of hematoxylin and eosin stained sections, the lesions studied ranged from mild dysplasia to infiltrating squamous carcinoma (Table 1). Thirty-five (83.9%) specimens included underlying stroma or substantia propria. Of

<table>
<thead>
<tr>
<th>Histopathologic grade</th>
<th>Number of specimens (%)</th>
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<tr>
<td>Squamous metaplasia/dyskeratosis</td>
<td>5</td>
</tr>
<tr>
<td>Mild dysplasia</td>
<td>3</td>
</tr>
<tr>
<td>Mild to moderate dysplasia</td>
<td>1</td>
</tr>
<tr>
<td>Moderate dysplasia</td>
<td>3</td>
</tr>
<tr>
<td>Moderate to severe dysplasia</td>
<td>5</td>
</tr>
<tr>
<td>Severe dysplasia/carcinoma in situ</td>
<td>12</td>
</tr>
<tr>
<td>Invasive carcinoma</td>
<td>11</td>
</tr>
<tr>
<td>Total</td>
<td>42</td>
</tr>
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</table>
Fig. 1. Raised limbal lesion in a patient who continued to harbor HPV-16 DNA bilaterally 8 years after this lesion was excised.

these, 25 (71.4%) exhibited histopathologic changes indicative of solar—presumably ultraviolet—exposure.

Bilateral limbal conjunctival swabs were obtained from five patients, and a unilateral swab of the affected eye was performed on one patient. Swabs were obtained intraoperatively in one patient and during a routine follow-up evaluation in five patients. The time between surgical excision of conjunctival lesions and performance of conjunctival swabs ranged from minutes (swab at time of surgery) to eight years. One patient was swabbed two months before surgery. Three (50%) of the patients from whom swabs were obtained were men. Five patients had tissue available for analysis, as well. The patient for whom no tissue was available was a woman who had had a limbal squamous lesion removed eight years earlier (Fig. 1).

DNA from HPV 16 was identified in 37 (88.1%) tissue specimens (Fig. 2). The four patients who had two separate surgeries had HPV DNA present in both samples. Of the 10 patients for whom multiple blocks existed from a single surgery, HPV DNA was identified in all blocks in seven (70%). Negative blocks were reviewed and found to contain no epithelium or epithelium that did not exhibit dysplastic change. In all cases, multiple blocks had been submitted to evaluate margins of tumor excision or to sample lesions elsewhere on the conjunctiva.

HPV 16 DNA was present in the one unilateral swab, and bilaterally in four of five (80%) patients (Fig. 3, Table 2). The one patient with negative bilateral swabs had a unilateral conjunctival squamous metaplasia evaluated in four separate paraffin blocks, all of which were positive for HPV 16 DNA. His conjunctivas were swabbed 20 weeks after surgical excision of the lesion. One patient whose swabs were positive one year after surgery developed a recurrence at 15 months postoperative. The recurrence contained HPV 16 DNA, as had the original lesion in this 86-year-old woman.

Controls of HPV 16 or 18 DNA were positive with appropriate type 16 or 18 primers and probes. Specimens of pterygia ligneous conjunctivitis and conjunctival melanoma were negative, as were samples processed with control DNA but no Taq, and with no template DNA.
We identified HPV type 16 DNA in 42 specimens of conjunctival squamous dysplasia or carcinoma. These findings provide additional evidence that conjunctival squamous neoplasia may be related to HPV, especially to the presence of HPV 16. The exact role of HPV in the development of such lesions remains unclear. Although HPV DNA was present in the vast majority of lesions, it was also present in tissue swabs from eyes with no visible lesions in four (66.7%) of six patients with unilateral conjunctival epithelial neoplasia. In one patient, HPV 16 DNA was present in the involved and uninvolved eyes eight years after excision of the lesion. Although HPV DNA could be isolated from swabs of both eyes of this patient, she did not develop a recurrence of tumor, and new lesions did not develop in the fellow eye.

Another patient had persistent bilateral positivity one year after surgery but went on to develop a recurrence at 15 months. The recurrence contained HPV 16 DNA, as did the original lesion and a recurrence at 7 years in another patient. These preliminary data, added to our previously reported case of bilateral swabs positivity in a patient with unilateral corneal dysplasia,9 raise questions about the exact role of HPV in the development of conjunctival squamous neoplasia.

Based on these results, HPV probably does not act alone in the development of conjunctival epithelial neoplasia. Classically, conjunctival squamous neoplasia is referred to as being of actinic origin.12 The theory that ultraviolet (UV) light plays a role in the development of these lesions is based on their position in the interpalpebral area and their predilection for older men,13 who are presumed to have a heavier exposure to sunlight than women. The presumed difference in sun exposure is an assumption not supported by data in any of the classic papers, yet it is used as an
explained for the disparity in occurrence of these lesions between men and women.

Clinical assessment of the degree of UV exposure is inaccurate, at best. Some authors have used employment, indoor versus outdoor, as a means for estimating UV exposure. Employment information was unavailable to us, but we did evaluate all specimens for histopathologic evidence of solar exposure. Twenty-five (83.3%) of 35 specimens exhibited solar elastosis, 21 (84%) of which also contained HPV 16 DNA. None of the 6 pterygia we examined—all of which, by definition, exhibit solar elastosis—contained HPV 16 DNA. In our patients, UV exposure alone does not explain the development of conjunctival epithelial neoplasia.

Nonetheless, UV light or some other element may interact with HPV in the development of conjunctival disease. The interaction of HPV with UV light has been postulated to play a role in the development of HPV-related tumors in the sun-exposed skin of patients with epidermolysis verruciformis.

Other factors also may interact with HPV in the conjunctiva or in other organ systems. In the female genital tract, the best-understood system for HPV-related neoplasia, there is a low but consistent increase in the relative risk of cervical carcinoma in women with a history of heavy or prolonged smoking. A recent case-control study examining risk factors for conjunctival intraepithelial neoplasia found no increased risk with sun exposure (measured by whether or not an individual was an office worker), but it did find a 3.8-fold increased risk of conjunctival epithelial neoplasia over age- and sex-matched controls among patients who smoked cigarettes. The authors did not evaluate the lesions or the control patients for the presence of HPV DNA. No conclusions can be drawn from these preliminary studies about whether other possible risk factors will affect outcome in patients with known ocular HPV infection.

The lesions we examined with PCR spanned the spectrum of severity from squamous metaplasia to infiltrating carcinoma. We examined all lesions in the files for which we had tissue, but most of the lesions were in situ or infiltrating carcinoma rather than less severe dysplasias. Although studies of the cervix have revealed that HPV typing of mild or moderate dysplasia holds little predictive value for outcome, not enough information is available about the conjunctiva to draw conclusions about whether HPV 16 positivity should change the management of a particular lesion. Although finding HPV 16 DNA in an affected eye may eventually offer clues, over and above the clinical impression, about the severity of the lesion, surgery remains the treatment of choice.

HPV-related anogenital lesions represent a sexually transmitted epidemic. How HPV gets to the conjunctiva is not clear. Can we expect that the increasing incidence of HPV infection of the anogenital tract will be followed by an increase in ocular disease, as has been the case with other sexually transmitted diseases such as chlamydia? What is the significance, if any, of ocular HPV infection, given our demonstration of HPV DNA in tumors as well as in grossly uninvolved eyes? These questions merit, and are receiving, additional study.

Key words: Conjunctival dysplasia/neoplasia, human papillomavirus, polymerase chain reaction, DNA

References


Table 2. HPV 16 DNA in conjunctival swabs

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<thead>
<tr>
<th>Patient</th>
<th>OD</th>
<th>OS</th>
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<tbody>
<tr>
<td>12*</td>
<td>+/+++ (R)</td>
<td>+/+++ (R)</td>
</tr>
<tr>
<td>13*</td>
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<td>++</td>
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<td>18</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>19</td>
<td>-</td>
<td>-</td>
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R = PCR studies repeated; both results shown; ND = Not done.
* Dot blot results shown in Figure 3.