

## SCIENTIFIC CORRESPONDENCE

## Human papillomavirus in pterygium

Arrow Park Hospital,  
Wirral, Liverpool, UK  
M J Gallagher

M J Gallagher, A Giannoudis, C S Herrington, P Hiscott

Molecular Pathology  
Department, Royal  
Liverpool University  
Hospital, Liverpool,  
UK

A Giannoudis  
C S Herrington

Department of  
Ophthalmic Pathology,  
Royal Liverpool  
University Hospital,  
Liverpool, UK  
P Hiscott

Correspondence to:  
Dr Michael J Gallagher  
Ophthalmic Department, St  
James's University Hospital,  
Beckett Street, Leeds  
LS9 7TF, West Yorkshire,  
UK  
mjgallagher@doctors.org.uk

Accepted for publication  
5 February 2001

Table 1 Details of specimens used

Specimen type	Case number	Age (years)	Sex	HPV type
Papilloma	1	39	M	6
	2	54	F	16
	3	58	F	6
	4	38	M	11
	5	81	M	11
	6	37	M	-
	7	87	F	6
	8	34	M	6
	9	24	F	11
	10	64	M	16
Pterygium	1	45	M	-
	2	72	F	-
	3	50	M	-
	4	31	F	11
	5	38	M	16
	6	57	M	11
	7	72	F	-
	8	61	F	-
	9	55	F	6
	10	68	M	6
Conjunctival primary acquired melanosis	1	60	M	-
	2	72	M	-
	3	42	M	-
	4	49	F	-
	5	78	M	-
	6	65	M	-
	7	60	F	-
	8	32	F	-
	9	51	F	-
	10	71	M	-
Conjunctival malignant melanoma	11	89	F	-
	12	74	F	-
	13	69	F	-
	14	37	F	-
	15	59	F	-
	16	53	M	-
	17	56	M	-
	18	75	F	-
	19	78	M	-
	20	51	M	-

## Abstract

**Aim**—To determine the prevalence and significance of human papillomaviral types in conjunctival pterygia.

**Methods**—Polymerase chain reaction technology was used to identify the presence of human papillomavirus (HPV) in 10 formalin fixed paraffin embedded pterygia samples. 10 conjunctival papillomas were used as positive controls. 20 conjunctival samples, 10 with primary acquired melanosis and 10 with malignant melanoma, were used as negative controls. Sample subgroups were of equal sex, race, and age distribution to eliminate bias. All samples were further analysed (for 21 HPV types) using dot-blot hybridisation techniques.

**Results**—HPV was identified in 90% of the conjunctival papillomas, 50% of the

pterygia samples, but no HPV was detected in the negative control group. Two pterygia showed type 6, two type 11, and one type 16. These three HPV types were also detected in papillomas.

**Conclusion**—These results suggest that HPV may be involved in the pathogenesis of pterygia and that broadly the same HPV types are found in pterygia and in papillomas. Persistent conjunctival HPV may possibly play a part in the recurrence of pterygia post excision but further larger studies are required to elucidate this hypothesis.

(Br J Ophthalmol 2001;85:782-784)

Human papillomaviruses (HPV) are composed of a closed circular double stranded DNA genome enclosed in an icosahedral capsid.<sup>1</sup> At least 80 different HPV genotypes are recognised, HPV 6 and 11 being associated with benign lesions and HPV 16 and 18 being closely linked with malignancy of the uterine cervical squamous epithelium.<sup>2</sup>

HPV is also associated with epithelial pathology elsewhere, including conjunctival papilloma<sup>3</sup> and conjunctival epithelial neoplasia,<sup>4</sup> and this pathology is manifest by changes such as alterations in epithelial thickness and dysplasia.

Pterygium is a chronic vision threatening disease of unknown origin and pathogenesis. It is characterised by the encroachment of a fleshy triangular portion of the bulbar conjunctiva onto the cornea, which is highly vascular and has an overlying squamous epithelium. The epithelium may exhibit a wide variety of degenerative and proliferative changes. Since some of the pathological features which can occur in the epithelium of a pterygium are similar to those induced by HPV in epithelia elsewhere, we investigated the possibility that HPV might be involved in the pathogenesis of pterygia.

## Methods

## CHOICE OF SPECIMENS

Specimens from 40 patients (21 male, 19 female) were analysed in the study. The specimens included 10 conjunctival papilloma and 10 conjunctival pterygia. Twenty conjunctival specimens, 10 with primary acquired melanosis and 10 with malignant melanoma, were used as negative controls. There was an equal sex distribution throughout all subgroups included (Table 1).

## DNA EXTRACTION

*Biopsies*

Three 5 µm formalin fixed, paraffin sections of biopsy material were dewaxed in xylene (2 × 5 minutes) followed by 96% ethanol (2 × 5 minutes) then pelleted and air dried for 1 hour. After overnight incubation at 42°C with 200 µg/ml proteinase K (Boehringer Mannheim) in 200 nM TRIS-HCl pH 8.0, 1 mM EDTA, the digest was heated to 95°C for 20 minutes to arrest enzyme activity. The supernatant was used in a 40 cycle polymerase chain reaction (PCR) reaction.

## β GLOBIN PCR

To analyse the quality of the target DNA, the samples were tested by β globin PCR, as previously described<sup>5</sup>; 20 µl of the extracted DNA were used in a 50 µl PCR reaction containing 50 mM KCl, 10 mM TRIS-HCl (pH 8.3), 200 µM of each dNTP, 1.5 mM MgCl<sub>2</sub>, 1U of *Taq* DNA polymerase, and 50 pmol of the PC03/PC05 primers amplifying a fragment of 209 bp. Samples were subjected to hot start at 95°C for 5 minutes and then 40 cycles of amplification using the following parameters: denaturation at 95°C for 1 minute, annealing at 55°C for 2 minutes, extension at 72°C for 1.5 minutes, followed by one cycle of extension at 72°C for 7 minutes. The PCR product was analysed on a 1.7% agarose gel stained with ethidium bromide.

## HPV PCR

This was performed as previously described.<sup>6</sup> Briefly, 20 µl of the extracted DNA were used in order to perform a 50 µl PCR reaction containing 50 mM KCl, 10 mM TRIS-HCl (pH 8.3), 200 µM of each dNTP, 3.5 mM MgCl<sub>2</sub>, 1 U of *Taq* DNA polymerase (Gibco), and 50 pmol each of the consensus GP5+/6+ primers. The primers amplify a fragment of 140–150 bp (depending on HPV type) from the L1 gene. Samples were subjected to 95°C for 4 minutes followed by 40 cycles of amplification using the following parameters: denaturation at 94°C for 1 minute, annealing at 40°C for 2 minutes, extension at 72°C for 1.5 minutes, followed by one cycle of extension at 72°C for 7 minutes to ensure a complete extension of the amplified DNA. The PCR product was analysed on a 1.7% agarose gel stained with ethidium bromide.

## HPV TYPING

This was performed as previously described.<sup>6</sup> Briefly, the PCR product was diluted 1:10 and denatured for 10 minutes at 95°C. One µl of the denatured DNA was applied on the nylon membrane and placed under ultraviolet light for 3 minutes, followed by baking at 80°C for 1–2 hours.

The membranes were placed into pre-hybridisation buffer (0.02% SDS, 1 g blocking reagent in 2 × SSC) for 1 hour at 55°C and hybridised overnight at the same temperature using individual 5'digoxigenin labelled oligonucleotide probes. Then, they were washed in post-hybridisation buffer (0.1% SDS in 2 × SSC) 3 × 10 minutes at 55°C and in blocking

solution (0.5% Tween 20, 1% blocking reagent in 1 × AP7.5) for 45 minutes at room temperature. Signal was detected using anti-digoxigenin-alkaline phosphatase conjugate diluted 1:5000 in blocking solution for 30 minutes. Finally, the membranes were washed in blocking solution 1 × AP7.5 (0.1M TRIS, 0.1M NaCl, pH 7.5, and 10 mM MgCl<sub>2</sub>) for 3 × 5 minutes and 1 × AP9.6 (0.1M TRIS, 0.1M NaCl, pH 9.6, and 10 mM MgCl<sub>2</sub>) and developed using nitroblue tetrazolium (NBT)/bromo-chloro-indolyl phosphate (BCIP) in AP9.6 (up to 16 hours).

**Results**

The papilloma group which acted as positive control demonstrated 90% positivity for the presence of HPV DNA. Five of the pterygia were also positive for HPV DNA. The negative conjunctival controls were entirely negative for the presence of HPV DNA. HPV typing demonstrated type 6 in four of the nine HPV positive papillomas (44%), type 11 in three samples (33%), and type 16 in two samples (22%). In the five HPV positive pterygia, HPV type 6 was found in two specimens (40%), type 11 in two specimens (40%), and type 16 in one specimen (20%). Two of the pterygia specimens were recurrent. However, neither were positive for the presence of HPV DNA. The clinical behaviour of the pterygia specimens demonstrated a normal growth pattern with no aggressive features seen.

Fisher's exact test incorporating Yates's correction was used to analyse the samples and indicates that pterygia have a significantly higher rate of positivity ( $p = 0.0017$ ) when compared with the normal samples.

**Discussion**

Our results confirm that HPV DNA can be detected in the vast majority of conjunctival papillomas and indicate that types 6, 11, and 16 may be involved in the pathogenesis of these lesions. Moreover, we found HPV DNA in half of the pterygia studied and that broadly similar HPV types were present in both papillomas and pterygia. Interestingly, in both lesions HPV type 6 was most commonly found and type 16 least often detected.

These observations raise the possibility that HPV may be involved in the pathogenesis of pterygia. Pterygia have been postulated to be caused by inflammation at the junction of the conjunctival blood vessels and Bowman's membrane where the autolytic process of inflammation results in a protein degradation amino acid mixture. It has been suggested that this amino acid mixture has the ability to attract conjunctival vessels onto the cornea.<sup>7</sup> Postulated causative agents of this process include members of the herpes virus family,<sup>8</sup> chronic ultraviolet exposure,<sup>9</sup> and other factors that cause repeated inflammatory insults to the interpalpebral corneoscleral junction (for example, dust and wind exposure). Recent evidence suggests that pterygia development may be a result of disruption of the normal process of apoptosis, the cells demonstrating significant levels of p53 as well as apoptosis inhibiting proteins.<sup>10</sup>

Conjunctival carcinoma has been associated with concomitant viral infection, principally human immunodeficiency virus (HIV), with HPV being implicated in a proportion of cases in an African study.<sup>11</sup> Viral-like intracellular inclusions have been described in a pterygium,<sup>12</sup> and Spandidos and co-workers have demonstrated herpes simplex virus (HSV) in 45% of pterygia.<sup>8</sup> However, HSV at the ocular surfaces usually causes a chronic inflammation with scarring and neovascularisation of the cornea. Conversely, HPV is associated with conjunctival epithelial pathology ranging from papillomas to squamous cell carcinoma.<sup>13</sup>

Conjunctival papillomas tend to recur after excision and such recurrence may reflect persistent viral infection. Pterygia also tend to recur after excision with recurrence rates of up to 46% after a 7 year follow up period.<sup>14</sup> This observation would suggest that if HPV is involved in the pathogenesis of these lesions, persistent conjunctival HPV infection may also lead to pterygium recurrence and that recurrent pterygia should be examined for HPV DNA. Moreover, if HPV has a role in pterygium recurrence, antiviral treatment might be used in the future to prevent this recurrence.

In conclusion, our findings of the presence of HPV DNA in pterygia supports the hypothesis that HPV may be involved in the epithelial changes seen in pterygia and that the virus may be causative in the pathogenesis of primary and recurrent pterygia. However, further larger studies are necessary to strengthen this hypothesis. It is possible that HPV may act synergistically with other causative agents representing a disease of multifactorial pathogenesis.

Presented as a poster at ARVO May 2000.

We thank the staff of the Molecular Pathology Laboratory, Duncan Building, Royal University Hospital, Liverpool, for all their help, and Mr Louis Clearkin, consultant ophthalmologist, Arrows Park Hospital, for supporting this project from his research fund.

- 1 Zur Hausen H. Human papillomaviruses and their possible role in squamous cell carcinomas. *Curr Top Microbiol Immunol* 1977;78:1-30.
- 2 Southern SA, Herrington CS. Molecular events in uterine cervical cancer. *Sex Transm Infect* 1998;74:101-9.
- 3 McDonnell PJ, McDonnell JM, Kessis T, et al. Detection of human papillomavirus type 6/11/2 DNA in conjunctival papillomas by in situ hybridisation with radioactive probes. *Human Pathol* 1987;18:1115-19.
- 4 McDonnell JM, McDonnell PJ, Yu Sun Y. Human papillomavirus DNA in tissues and ocular surface swabs of patients with conjunctival epithelial neoplasia. *Invest Ophthalmol Vis Sci* 1992;33:184-9.
- 5 Southern SA, Evans MF, Herrington CS. Basal cell tetrasomy occurs in low grade cervical squamous intraepithelial lesion infected with high risk human papillomaviruses. *Cancer Res* 1997;57:4210-13.
- 6 Giannoudis A, Evans MF, Southern SA, et al. Basal keratinocyte tetrasomy in low-grade squamous intraepithelial lesions of the cervix is restricted to high and intermediate risk HPV infection but is not type-specific. *Br J Cancer* 2000;82:424-8.
- 7 Wong WW. A hypothesis on the pathogenesis of pterygia. *Ann Ophthalmol* 1978;10:303-8.
- 8 Spandidos DA, Xinarianos G, Ergazaki M, et al. The presence of herpesviruses in pterygium. *Int J Oncol* 1994;5:749-52.
- 9 Coroneo MT. Pterygium as an early indicator of ultraviolet insolation: a hypothesis. *Br J Ophthalmol* 1993;77:734-9.
- 10 Tan D, Tang W, Liu Y, et al. Apoptosis and apoptosis related gene expression in normal conjunctiva and pterygium. *Br J Ophthalmol* 2000;84:212-16.
- 11 Waddell KM, Lewallen S, Lucas SB, et al. Carcinoma of the conjunctiva and HIV infection in Uganda and Malawi. *Br J Ophthalmol* 1996;80:503-8.
- 12 Yanoff M, Fine BS. Cornea and sclera. In: *Ocular pathology. A text and atlas*. Hagerstown, 1975:261-324.
- 13 Karcioglu ZA, Issa TM. Human papilloma virus in neoplasia and non-neoplastic conditions of the external eye. *Br J Ophthalmol* 1997;81:595-8.
- 14 Allan BDS, Short P, Crawford GJ, et al. Pterygium excision with conjunctival autografting: an effective and safe technique. *Br J Ophthalmol* 1993;77:698-701.