EXTENDED REPORT

Detection of human papillomavirus DNA in pterygia from different geographical regions

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Background/aims: The aetiology and pathogenesis of pterygia remain unclear and the involvement of human papillomavirus (HPV) is controversial. 41 pterygia from two geographic locations were evaluated for the presence of HPV DNA.

Methods: 41 pterygium biopsies (17 from Italy and 24 from Ecuador) were analysed using the L1C1 and PU-1ML primer sets by polymerase chain reaction (PCR) and DNA sequence analysis.

Results: 22 of the 41 pterygia (54%) were positive for HPV, including all 17 Italian cases and 5/24 (21%) Ecuadorian cases. DNA sequencing of the 22 positive cases showed that 11 were HPV type 52, four were type 54, five were *cand*HPV90, and two of unknown genotype.

Conclusions: The major differences in the frequency of HPV in geographically distant populations might suggest a possible explanation for the vast differences in the reported detection rates. Three subtypes of HPV were found in this sample of pterygia. None the less, these results suggest that HPV may have a pathogenic role in pterygium.

Human papillomaviruses (HPV) are involved in the aetiology of benign and malignant neoplasia of cutaneous and mucosal epithelia, with more than 130 types identified.¹ Among these, more than 100 types may have a role in human cutaneous neoplasias² and more than 40 types have been associated with anogenital cancers.^{3–7} HPVs may also be involved in conjunctival papilloma^{8–9} and conjunctival epithelial neoplasia.¹⁰

Pterygium is a chronic condition consisting of a superficial growth of a highly vascularised loose connective tissue covered by an epithelium, which shows, alternately, thickening or thinning with elastoid and basophilic degeneration of the underlying connective tissues.^{11–12}

The aetiology and pathogenesis of pterygium remain unclear. Several hypotheses have been proposed including inflammatory reactions,^{13–14} a primary degeneration of the cornea followed by fibroblastic proliferation,^{15–16} allergic factors,¹⁷ and immunopathological mechanisms.^{18–19} However, the finding of molecular anomalies in pterygia, including loss of heterozygosity (LOH) at loci near various tumour suppressor genes, as well as increased expression of p53, suggests that this pathology may be neoplastic in nature.²⁰

Epidemiological studies indicate that chronic exposure to the sun and most probably ultraviolet B light (UV-B) is an important factor in the development of pterygia. Several surveys have shown that countries nearer the equator have higher rates of pterygia, with a 36-fold increased risk for people with early, intense exposures (residing at 30° S latitude or less) for the first 5 years of life.²¹ UV-B has been shown to activate viruses such as herpes, HIV, and human papillomaviruses.^{22–24} Moreover, UV-B radiation has been found to be mutagenic for the p53 gene,^{25–26} which was found to be overexpressed in the limbal basal stem of all pterygia and limbal tumours and most pingueculae from which these growths seem to originate.²⁷ This could indicate the probable existence of p53 mutations in these lesions, although none has been detected to date.²⁸ In pterygia, apoptotic cells are mainly confined to the basal portion of the epithelial layer and express significant levels of p53, bax, and bcl-2. This is in contrast with normal conjunctival specimens and supports a model wherein development of pterygia is a result of disruption of the normal process of apoptosis occurring in the conjunctiva.²⁹

At present, the involvement of HPV in the genesis of pterygium is controversial, as some authors have reported that HPV is present in up to 50% of cases, while others have failed to detect HPV in pterygium.^{30–34} In an effort to resolve this dilemma, we evaluated a series of 41 pterygia derived from two different geographic locations for the presence of HPV DNA by polymerase chain reaction (PCR) with three different primer sets. This was accompanied by direct DNA sequence analysis in order to determine the viral genotype.

MATERIALS AND METHODS

All the studies performed were approved by the respective ethics committees.

Tissues

Human biopsies of pterygium were obtained from 41 patients—17 from the Department of Ophthalmology, University of Cagliari and 24 from the Department of Pathology, Cancer Center of SOLCA, Cuenca, Ecuador. In both institutions, the biopsies were fixed in 10% buffered formalin, pH 7.3, and paraffin embedded.

PCR and DNA sequence analysis for HPV

Three to five paraffin sections (5 µm) were dewaxed in xylene, followed by 95% ethanol, pelleted, air dried, and incubated at 40°C overnight in 50 µl of 100 mM TRIS-HCl, pH 8, 1 mM EDTA, 1% Tween 20, and 200 µg/ml proteinase K. After incubation at 95°C for 7 minutes, 2 µl of the supernatant was used for PCR. Three different primer sets were used: (1) forward primer L1C1, reverse primers L1C2 and L1C2M³⁵; (2) forward primer PU-1M-L, reverse primers PU-2R and PU-2RN,³⁵ and (3) MY09 and MY11.³⁶ PCR was performed on 20 µl of 50 mM KCl, 10 mM TRIS-HCl pH 8.3, 0.2 mM dNTP, 4 mM MgCl₂, 1 U of Taq, and 0.5 µM of each primer. When three primers were used, the concentration of the two reverse primers was 0.25 µM each. Forty cycles of amplification were performed using the following cycles: 95°C for 1 minute, 58°C for 1 minute, and 72°C for 1 minute. The PCR product was sequenced using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems).

Table 1 HPV genotypes found in pterygia

	Virus type*	% Identity
Italy		
I-16	<i>candHPV90</i>	90
I-19	HPV52	96
I-22	HPV52	99
I-24	HPV52	95
I-25	<i>candHPV90</i>	92
I-26	HPV52	94
I-28	<i>candHPV90</i>	97
I-29	HPV54	96
I-33	HPV52	93
I-40	HPV52	96
I-41	HPV52	95
I-42	HPV54	97
I-43	ND	
I-48	HPV52	91
I-49	<i>candHPV90</i>	94
I-57	HPV52	90
I-59	HPV52	99
Ecuador		
E-1	HPV54	92
E-2	HPV52	92
E-3	HPV54	93
E-4	ND	
E-10	<i>candHPV90</i>	96

*ND = not determined.

Controls

To verify the efficacy of the L1C1 primer set under our PCR conditions, we compared this primer set with the MY09/MY11 primer set, currently in routine use in our molecular diagnostics laboratory, on a panel of 57 cervical biopsies for which clinicopathological data are available. To verify the quality of the target DNA and the absence of PCR inhibitors, β actin primers were used in a control PCR.

RESULTS

Detection of HPV DNA in cervical biopsies and choice of primers

The primer sets L1C1 and MY09/MY11 amplify a region from L1 gene of about 250 and 450 bp, respectively. To verify the validity of our PCR conditions, we first analysed a panel of 57 cervical biopsies in a double blinded fashion using both primer sets. Using the former set, 37 cases tested positive while with the latter primer set a total of 34 cases were positive. After decoding, these data were shown to also be in agreement with available histopathological data—that is, each of the 15 cases showing koilocytosis were positive for viral DNA and the remaining 22 HPV positive cases included 14 low grade squamous intraepithelial lesions (L-SIL) and eight had only inflammatory changes. Thus, as previously noted, the L1C1 primer set detects a broad spectrum of HPV viral subtypes.³⁵ Given the slightly higher detection rate using the L1C1 primer set, it was chosen for initial evaluation of pterygium.

Detection of HPV DNA in pterygium

Seventeen of the 41 pterygia were from Italy and 24 were from Ecuador. Using the L1C1 primer set, 22/41 (54%) were positive for the virus (Table 1). Typical examples are shown in Figure 1. All Italian cases were positive (100%), while only 5/24 (21%) cases from Ecuador were HPV positive. β Actin was successfully amplified in all cases. To confirm these results, a second primer set (pU-1M-L/pU-2R) was used which amplifies a sequence of about 250 bp in the E6/E7 region. Identical results were obtained using these primers. The MY09/MY11 failed to amplify a PCR product in all but two cases. However, the MY09/MY11 amplicon is somewhat larger than the L1C1 product, which is undoubtedly less suited to amplify the par-

Figure 1 Representative examples of PCR amplification using the L1C1 primer set and DNA sequence analysis of HPV from biopsies of pterygia. In (A) the HPV amplified PCR product is approximately 250 bp in length. In (B) the PCR amplification of β actin sequences is shown as a control. In (C) and (D) the DNA sequences of a HPV52 and *candHPV90* genotypes are shown, respectively.

tially degraded DNA present in formalin fixed, paraffin embedded samples.

DNA sequence analysis of PCR amplified HPV products

The 22 PCR products amplified using the L1C1 primer set were subjected to DNA sequence analysis and subsequent BLAST searching. Eleven of the positive specimens were found to be HPV type 52, five were type 54, and six were a newly identified *candHPV90* with percentages of identity varying from 90% to 99%. Ten of the PCR products amplified from cervical biopsies were also sequenced revealing various genotypes that included 16, 18, 32, 51, 52, 58, and 70. The MY09/MY11 primer set will not amplify HPV types 52 and *cand90*, explaining the negative results in pterygia using this primer set.

DISCUSSION

Only three studies have addressed the presence of HPV DNA in pterygia, all using PCR amplification. A study from Greece identified HPV in 15 of 50 cases (30%), which, using type specific primers, was reported to be HPV type 18 in all cases.³¹ A study from the United Kingdom found that five of 10 cases were positive and that the genotypes involved were 6, 11, and 16 by dot-blot hybridisation.³³ The third study from the United States reported that all 13 cases studied were HPV negative.³⁴ One possible explanation for these differences might be that HPV is present in pterygium at different frequencies in geographically distinct populations.

To address this issue, we analysed pterygia from two geographically distant locations for the presence of HPV using PCR with different primer sets, the validity of which was directly demonstrated using a panel of cervical biopsies. HPV was detected in 100% of Italian pterygia and in 21% of those from Ecuador. Moreover, direct sequence analysis identified three strains of HPV in pterygium comprising HPV 52, *candHPV90*, and HPV 54.

Our data would substantiate the hypothesis of the existence of geographical differences in the frequency of HPV involvement in pterygium. This may also suggest that different lifestyles, environmental conditions, and genetic make up also affect the presence and pathological contribution that HPV could have in pterygium.

It is of further interest that only three subtypes of HPV were found in our panel of pterygia. Others have reported the presence of other genotypes in pterygium, including the low risk

types 6 and 11 and the high risk types 16 and 18.³¹⁻³³ Our data, although limited, do not suggest that type specific geographic differences exist, as the same three HPV subtypes were found in pterygia from both Italy and Ecuador. Moreover, the HPV genotypes in our panel of cases (HPV 52, 54 and the recently discovered *cand90*) are in agreement with the benign low risk nature of these lesions.

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