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Research article

Human papilloma virus presence and its physical status in primary pterygium

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ABSTRACT

Pterygium is one of the most frequent pathologies in ophthalmology, and is a benign, overgrowth of fibrovascular tissue, often with a wing-like appearance, from the conjunctiva over the cornea. It is composed of an epithelium and highly vascular, sub-epithelial, loose connective tissue. There is much debate surround the pathogenesis of pterygium and a number of theories have been put forward including genetic instability, cellular proliferation, inflammatory influence, and degeneration of connective tissue, angiogenesis, aberrant apoptosis and viral infection. At present, the involvement of human papillomavirus (HPV) in the genesis of pterygium is controversial, as have reported that HPV is present in 58% of cases, while others have failed to detect HPV in pterygium. In this study, we evaluated the presence and viral genotype of HPV DNA in pterygia and healthy conjunctiva sample, and virus integration into the cellular genome.

Forty primary pterygia samples and 12 healthy conjunctiva samples were analyzed to HPV DNA presence by polymerase chain reaction, using MY09/MY11 primers of HPV-L1 gene. Viral genotype was identified by DNA sequence analysis of this amplicon. HPV integration into the cellular genome was analyzed by western blot detecting HPV-L1 capsid protein.

Presence of HPV was observed in 19 of the 40 pterygia samples. In contrast, healthy conjunctiva samples were negative. To determine virus type, sequence analyses were performed. Interestingly, 11 out of the 19-pterygium samples were identified as HPV-11 type, meanwhile, the remaining 8 pterygium samples were identified as HPV-18. HPV-L1 capsid protein were found only in 3 out of the 10 samples studied.

In conclusion, our study identified the presence of HPV DNA exclusively in pterygium samples and described HPV-11 and -18 genotypes. Our results suggest that HPV may be involved in the pathogenesis of pterygium. On the other hand, the expression of the L1-HPV protein suggests viral integration into the cellular genome.

Abbreviations: HPV, Human Papilloma Virus; UV, Ultra Violet Radiation; OSSN, Ocular Surface Squamous Neoplasia.

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1. Introduction

Pterygium is an overgrowth of fibrovascular tissue, with a wing-like appearance, from the conjunctiva over the cornea [1–4] that have a predilection for nasal limbus and their growth can obscure the visual axis and can cause irregular astigmatism and chronic inflammation [5,6]. Pterygium pathophysiology is characterized by invasion of the basement membrane of normal cornea with the concomitant dissolution of Bowman's layer [7] which is highly vascular and has an overlying squamous epithelium; that means that some of the pathological features which can occur in the epithelium of a pterygium are similar to those induced by HPV in epithelia elsewhere [8]. Although the pathogenesis of pterygium is not clearly understood, certain findings concerning common features in pterygium and neoplasia have been proposed, raising the possibility that a pterygium is a neoplastic-like growth disorder [9].

Several theories have been postulated for the pathogenesis of pterygium, including immunological mechanisms, infections, and ultraviolet exposition [8,10,11], the precise basis by which pterygium is caused remains still under study. In this context, some studies indicate that chronic exposure to ultraviolet (UV) radiation is an important factor in the development of pterygium [12,13]. It has been proposed that UV irradiation has a carcinogenic effect, resulting in DNA damage with loss of normal growth control [14,15] and activation of viruses such as herpes, HIV, and human papillomaviruses [16].

However, it seems that UV irradiation is not the only cause to origin of pterygium. It has been proposed a model to explain pterygium development, known as "two-hit" model [17]: the first hit is an event mediated by UV exposure generating damage that causes genetic alteration or mutations, and the second hit is mediated by a viral infection, in susceptible cells.

Human papillomavirus (HPV) can cause this infection and promote tumor development. Depending about different HPV subtypes which are associated with benign or malignant epithelial tumors; for example, HPV 6 and 11 are associated with benign neoplasia while HPV 16 and 18 are closely linked with malignancy of the uterine cervical squamous epithelium [18]. On the other hand, HPV is also associated with epithelial tumors of the conjunctiva, including conjunctival papilloma [19] and conjunctival carcinoma. (McDonnell et al., 1992) [20]. However, the involvement of HPV in the genesis of pterygium is controversial, since some authors have reported that HPV is associated in 100% of cases, others have failed to detect HPV in pterygium [8,16,21–32]; McDonell et al., 1992; [13,20]. In this study, we evaluated the presence and viral genotype of HPV DNA by polymerase chain reaction in pterygia and healthy conjunctiva sample, while the possible virus integration into the cellular genome was determined by western blot.

2. Materials and methods

2.1. Biological samples

This study was conducted at Instituto de Oftalmología "Fundación de Asistencia Privada Conde de Valenciana" I.A.P. in Mexico City, Mexico. We included forty nasal primary pterygium samples obtained from 40 patients who underwent surgical resection and had histopathological confirmation of an epithelium without dysplasia, in order to include a patient without any other oncogenic or HPV related disease. Exclusion criteria for both groups were being younger than 18 years-old and having an OSSN recurrent lesion. Our study followed the tenets of the Declaration of Helsinki and was approved by our institutional ethics board. All patients agreed to participate and provided written informed consent.

2.2. DNA extraction and PCR analysis

Immediately after surgical procedure, the collected samples were snap frozen and kept at −80 °C until processed. Isolation of genomic DNA from biological specimens was performed using the QuickGene-810 semiautomatic DNA extraction system, following the manufacturer's instructions (Autogen, Holliston, MA, USA). Briefly, tissues were homogenized tissues using Tissue-Ruptor (Qiagen, Hilden, Germany), and were added to specific hydrophilic cartridges, avoiding nuclease contamination in a sealed space. DNA isolated was recovered in sterile tubes and after that was quantified in a NanoDrop 2000 (ThermoScientific, USA). At least 100 ng per sample was used as starting DNA material.

In order to identify the presence of HPV in biological samples, PCR assays were performed using MY09 (5′-cgtccmarrgcawactgatc-3′)/MY11 (5′-gcmcagggwataayaatgg-3′) primers of *HPV-L1* gene [33,34]; DNA extracted from HeLa cells was used as a positive control for HPV amplification; the *18s* gene was used as a housekeeping gene [35]. PCR assays without DNA template were performed as (Non-template controls, NTC) negative controls for the PCR reaction. All PCR reactions were achieved for 35 cycles in an Axygen Maxygen apparatus (Axygen Scientific Inc., CA, USA). PCR amplification conditions were: 94 °C for 60s; 55 °C for 60s and 72 °C for 60s. PCR amplification products were resolved in 1.5% agarose gels and stained with ethidium bromide; the observed bands with the amplicons were excised and the DNA was subsequently purified using the Qiaex II kit (Qiagen, Hilden, Germany) for DNA sequencing.

2.3. DNA sequencing

PCR products were cleaned up with centrisep columns (Applied Biosystems, Foster, CA) and were directly sequenced with dye termination chemistry by means of the BigDye Terminator Cycle Sequencing System using an ABI PRISM 3130 DNA Analyzer (Applied Biosystems Inc, USA). DNA sequences were subsequent BLAST searching (http://blast.ncbi.nlm.nih.gov/).

2.4. Extraction and quantitation of tissue proteins

Immediately after surgical excision, specimens were placed in ice-cold lysis buffer [20 mM Tris pH 7.5; 1 mM EDTA; 0.15 M NaCl; 50 mM NaF; 1% Triton X-100; 4 mM Na_3VO_4 and protease inhibitor cocktail tablets (Roche, Mannheim, Germany)]; after that, tissues were homogenized using Tissue-Ruptor (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The proteins were quantified by the DC Protein Assay kit (Biorad, California, USA) and were kept at −80 °C until use.

2.5. Western blot analysis

Sixty micrograms of proteins obtained from each pterygium and healthy conjunctiva were loaded onto 12% SDS-PAGE. After electrophoresis, the proteins were then transferred to a nitrocellulose membrane (BioRad, California, USA); nonspecific binding was blocked incubating for 1 h at room temperature (RT) with 5% non-fat milk diluted in PBS-Tween 20 (0.1%). Membranes were incubated overnight at 4 °C with mouse monoclonal anti-HPV-L1 (Bio-SB, CA, USA) or rabbit polyclonal anti-GAPDH (SantaCruz Biotechnology, Santa Cruz, CA). The membranes were then incubated with biotin anti-mouse and anti-rabbit conjugated antibodies (Jackson ImmunoResearch, West Grove, PA) for 2 h at RT. Finally, the membranes were incubated with peroxidase-anti-biotin conjugated antibodies (Roche, IN, USA) for 1 h at RT. Enhanced chemiluminescence reagent (GE, Piscataway, NJ, USA) was used to develop the reaction. Chemiluminesence was visualized and digitalized with G-Box Diversity System (Syngene, London, UK).

2.6. Statistical analysis

Data obtained were analyzed by chi-square test, a $p < 0.05$ was considered statistically significant. (Prism 9, GraphPad, San Diego, CA).

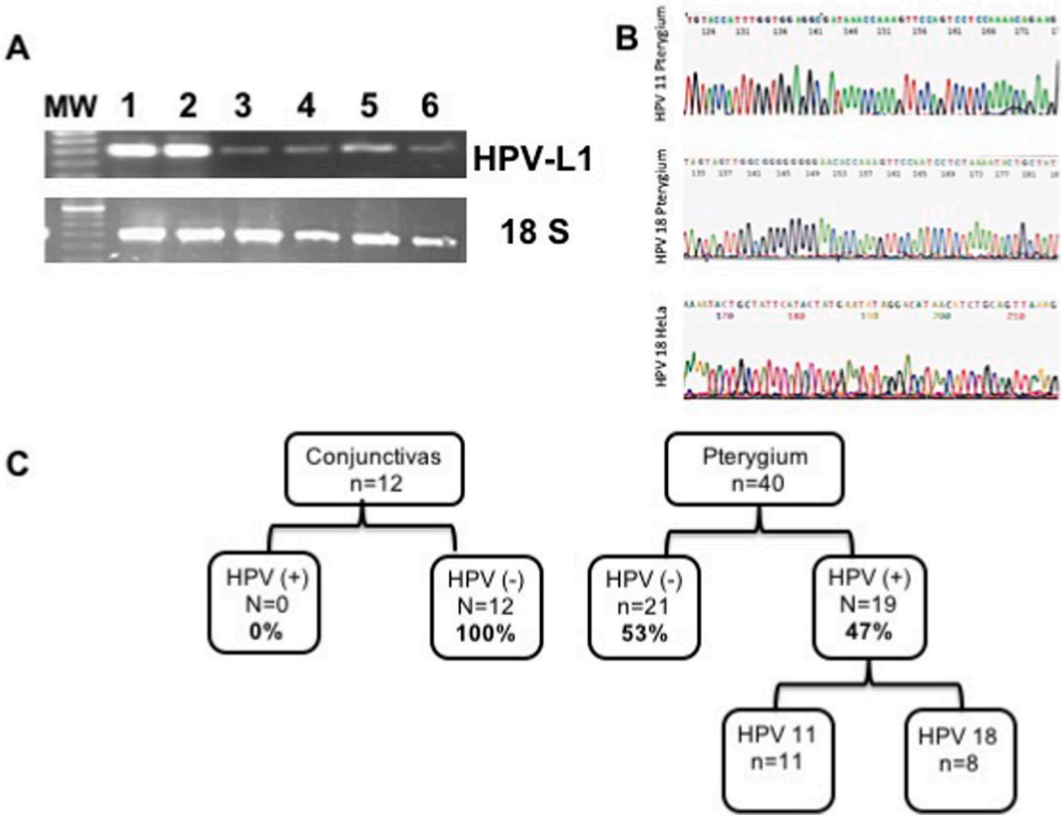

Fig. 1. HPV-11 and -18 types were present in pterygium samples. PCR amplified HPV products were sequenced. DNA sequences were subsequent BLAST searched (http://blast.ncbi.nlm.nih.gov/) and analyzed to identify HPV type. A) Representative samples of HPV-L1(a) and 18S (b) of PCR amplification of HeLa cells (as a control, lane 1) and HPV from pterygia samples (lanes 2–6). B) The DNA sequences of HPV11 and HPV18 genotypes from HeLa cells and pterygium are shown. C) Flow chart showing number of pterygia samples analyzed and the frequency of HPV genotypes identified.

3. Results

3.1. HPV DNA was exclusively detected in pterygium samples

The Presence of HPV was analyzed by PCR in pterygium and healthy conjunctiva samples using MY09/MY11 primers. A band of 450 bp was observed in 19 out of the 40 analyzed pterygium samples, which corresponded to the predicted size amplicon. In contrast to pterygium samples, this band was absent in all the healthy conjunctiva specimens (*p = 0.0027 Chi-square = 8.98; p = 0.0079; Yates correction = 7.05). In order to determine that DNA of the samples was sufficient and preserved, *18s* control gene was amplified. A band of 299 bp was observed in all the tissue samples, which corresponded to the predicted size amplicon of *18s* DNA (Fig. 1A). The patients recruited for this study showed an age range of 50–69 years, a mean of 59.5 ± 5 years; sixty three percent of the patients were female and 37% were male.

3.2. DNA sequence analysis of PCR amplified HPV products

In order to determine the molecular identity, sequence analyses and subsequent BLAST searching of the 450 bp amplicons that were exclusively obtained from pterygium samples were performed. Interestingly, 11 out of the 19-pterygium samples were identified as HPV-11 type, meanwhile, the remaining 8-pterygum samples were identified as HPV-18. Fig. 1B and C summarizes the results of the identity of each one of the amplicons sequenced and analyzed.

3.3. HPV-L1 protein identification by western blot

To verify the presence of virions localized in tissue samples and possible virus integration into the cellular genome, western blots assays identifying HPV-L1 capsid protein in pterygium samples were performed. Ten samples with enough quantity of protein were selected out of the 19 positive HPV-DNA containing pterygia-specimens were chosen to be analyzed by western blot. Six samples were positive to HPV-11 and 4 were positive to HPV-18. Interestingly, only 3 out of the 10 samples were positive to HPV-L1 capsid protein, two of these samples were positive to HPV-11 and the other was positive to HPV-18. Meanwhile, GAPDH that was used as a loading protein control was identified in all samples (Fig. 2).

4. Discussion

Pathogenesis of pterygium has been associated with several risk factors, including immunological mechanisms, ultraviolet exposition, oxidative stress, and viral infections. Among virus that has been described in pterygium, HPV is one of the most common found in this pathology; several studies have analyzed the presence of this virus in pterygia and has been thought by autoinoculation from contaminated fingers [36]. However, results of these studies are controversial; some of them described the presence of HPV in all the samples analyzed, other authors did not find HPV DNA in pterygia samples [16,21]; Rodrigues et al., 2008; [8,22–32,37]; McDonell et al., 1992; [13].

In this study, we evaluated the presence of HPV DNA by polymerase chain reaction and viral genotype was identified by DNA sequence a meanwhile analysis in pterygia and healthy conjunctiva samples. Our results showed the presence of HPV DNA in 47% of primary pterygia samples, very similar to the presence of HPV DNA in 48.9% reported in the Malay Peninsula [38]; and the absence of HPV DNA in healthy conjunctival samples. Sequence analysis from positive samples identified HPV 11 and HPV 18 types. In fact, in both primary pterygium samples HPV type 11 was most commonly found and type 18 least often detected. These observations support the possibility that HPV may be involved in the pathogenesis of pterygia. On the other hand, western blot analysis showed the presence of capsid protein from HPV.

In our study we have identified the presence of HPV DNA exclusively in pterygium samples as reported by others (Tsai, 2009) [39]. In contrast to them who found a lower association between HPV and pterygium, the prevalence of HPV associated with pterygium was higher in our study population; these prevalence differences could be related to external factors like the environment. Our data would substantiate the hypothesis of the existence of geographical differences in the frequency of HPV involvement in pterygium. Piras et al., analyzed pterygia from two geographically distant locations for the presence of HPV using PCR. HPV was detected in 100% of Italian

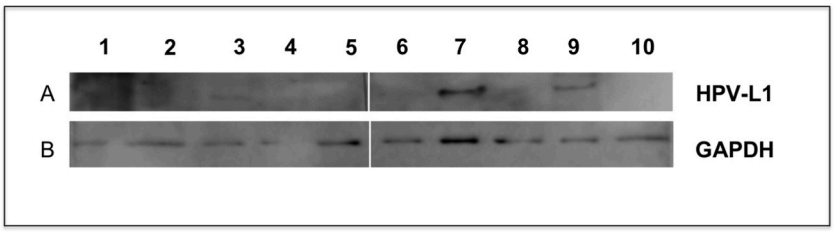

Fig. 2. Identification of HPV-L1 protein in pterygia samples. Sixty micrograms of total protein from ten pterygia were analyzed by western blot. A) HPV-L1 protein in pterygia samples was identified (57 kDa). B) GAPDH was used as a loading control.

pterygia and in 21% of those from Ecuador. This may also suggest that different lifestyles, environmental conditions, and genetic background also affect the presence and pathological contribution that HPV could have in pterygium. Moreover, the frequency of High Risk-HPV-18 in our study (42%) is high, in contrast with other reports (Detorakis et al., 2001; [40,41]; however, this high frequency in samples of our study is in concordance with the distribution of HPV genotypes in Mexico, where the prevalence of HPV is 20.4%, and around of 32% of the women in the world have HPV-16 and -18 DNA [42], and 3% and 1.7% in male for HPV-16 and -18 respectively [43].

On the other hand, pterygium is considered a neoplastic disorder, showing morphological features such as an uncontrolled cellular proliferation process and alterations in suppressor tumor genes as p53 and p27. It has been shown a relation between UV radiation and oncogenic viruses such as probable mutagenic factors for the p53 gene that lead to abnormal expression in the limbal basal stem of all pterygia and limbal tumors [44].

Currently, there is no very clear knowledge how HPV is involved in the development of pterygia. But what it is known is that HPV is composed by a closed circular double-stranded DNA genome with 8 open reading frames which are divided into 3 functional regions: The early (E) region encoding proteins E1- E7 responsible for viral replication, the late (L) region encoding structural proteins L1-L2, and the long control region (LCR) responsible for transcription and replication, and only the E6 and E7 proteins of high-risk HPV (HR-HPV) strains present transforming properties by interacting with the tumor suppressor genes p53 and with the retinoblastoma family of proteins pRb, involved in controlling cell proliferation [5]. It is important to mention that some main function of HR-HPV E6 onco-protein is to bind to p53 and induce degradation and inactivation of wild-type p53. Chong et al., showed in their study that pterygium patients are indeed infected by HR-HPV and considered that the increased expression of mutant p53 in pterygium without apoptosis is an indication of HPV involvement through E6 [38].

It has been described that UV is one of the most important agents in generate reactive oxygen species (ROS), and this could affect the expression or generating mutation of p53, and this is one of the hypotheses about pterygium development [44]. As we mentioned above, HPV-E6 is also associated with the degradation/inactivation of p53 (Chakia et al., 2019). Overexpression of peroxiredoxin 2, an antioxidant enzyme, is proposed that could inhibit the apoptosis induced by oxidative stress in pterygium cells [45]. Taken together these reports, UV, HPV and oxidative stress are related with alterations of p53. Detorakis et al. proposed "two hits hypotheses" in the development of pterygium, where the first hit is mediated by UV exposition causing genetic alterations into conjunctival cells, and the second hit is an oncogenic factor mediated by viral infection, as HPV. In the middle of this proposal, oxidative stress is an important factor that could link these two factors with p53 damage. Nevertheless, HPV, UV and p53, by themselves they are not the only ones responsible for the development of the pterygium, however, the association of more than one could contribute to pterygia growth.

HPV consists of an icosahedral capsid, primarily consists of L1 proteins, which confer viral entry ability. Moreover, expression of the L1 capsid protein has also been shown to be prognostic and a key therapeutic target [46]. To our knowledge this is the first report identifying HPV DNA and L1-HPV protein expression by western blot in pterygium samples.

The detection of HPV-L1 protein is associated with the productive viral status into the cell. Which suggests that when this protein is not identified, the virus DNA integrates to the host DNA and the upstream regulation sequence of HPV L1 gene is lost because of the rearrangement of virus genome, which suppresses the L1 gene transcription [47].

Hernandez et al., in their study about cervical intraepithelial lesions suggested that the lack of L1 expression may identify patients at higher risk for progression to squamous cell carcinomas (SCC), while expression of the L1 protein may identify lower risk lesions. In our study L1-HPV protein was found in 3 out of 10 representative pterygia samples analyzed. In fact, two of these samples were positive to HPV-11, the other was positive to HPV-18 and the rest did not express. Therefore, the evaluation of HPV L1 expression may be relevant for prognosis about lower and higher risk lesions, depending on the identification of HPV and determination of its oncogenic potential. However, another methodology must be performed in order to analyze HPV integration sites into the host cell, as Kamal S et al. identified different integration signatures using HPV double capture and next-generation sequencing (NGS) [48].

The HPV types can be divided according to their potential ability to cause neoplastic lesions. Those low-risk oncogenes (6, 11, 42, 43, 44) and those high-risk oncogenes (16, 18, 31, 45, 56) (Piecyt et al., 2009) [49]. In our study, HPV infections of high oncogenic risk were found in 8/19 of cases, while infections with HPV of low oncogenic potential that are the most commonly identified in conjunctival papillomas, were found in 11/19 of cases [36]. There is nevertheless some hope to pterygium that vaccines, antiviral drugs or gene therapies may eradicate or prevent their development [40]. However, further studies are required to improve the identification of HPV-L1 by PCR using a combination of GP5+/6+ and PGMY09/11 primers sets and nested PCR in order to detect more HPV-types. Even the detection of HPV-L1 protein by western blot must be evaluated using another antibody against this capsid protein of HPV directed to different peptide sequences of this protein, trying to avoid a false negative result.

In conclusion, our study identified the presence of HPV DNA exclusively in pterygium samples. Our results suggest that HPV may be involved in the pathogenesis of pterygium, in relation to external factors such as ultraviolet exposition and geographical differences. On the other hand, the expression of the L1-HPV protein may be suggesting viral integration into the cellular genome, as well as be relevant for prognosis about lower and higher risk lesion.

Author contribution statement

Leslye Sámano Hernández: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Y Garfias; Humberto González-Márquez: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Corazón-Martínez LA: Analyzed and interpreted the data.

Victor Bautista-de Lucio: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents,

materials, analysis tools or data; Wrote the paper.

Data availability statement

Data will be made available on request.

Additional information

No additional information is available for this paper.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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