Frequency of Human Herpesvirus 6 (HHV-6) in Pterygium Using Real-Time PCR Based on SYBR-Green I Fluorescence

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ABSTRACT

Background and objectives: Pterygium is a non-cancerous growth of conjunctival tissue that can extend onto the corneal surface. The presence of some oncogenic viruses in pterygium and the neoplastic nature of these lesions led us to the postulated involvement of the viruses in the etiology of pterygium. Given the association of human herpesvirus 6 (HHV-6) with ocular diseases, we aimed to investigate presence of this virus in pterygium.

Methods: Fifty tissue specimens were collected from patients with pterygium who underwent pterygium surgery between February 2013 and May 2015. The specimens were tested by real-time PCR using Maxima SYBR Green/ROX qPCR Master Mix (2X) kit. Demographic and clinical data were collected and analyzed using SPSS software (version 18).

Results: Six (12 %) specimens were positive for HHV-6 DNA. There was no statistically significant correlation between pterygium and presence of HHV-6.

Conclusion: Based on the results, a direct association between HHV-6 and development of pterygium seems less probable, which suggests that other etiologic agents must be involved in the multistep process of the disease.

Keywords: Human Herpesvirus 6; pterygium; Real-time PCR.

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INTRODUCTION
Pterygium is a common ocular lesion that appears as wing-shaped, vascular and fleshy growth that can extend onto the corneal surface. The disease is relatively common in the general population and thought to be caused by ultraviolet-light exposure, low humidity and dust (1). Much attention has recently been paid to the possibility of viral oncogenes involvement in the multistage process of pterygium formation. Until now, a few viruses such as human papillomaviruses (HPV) and Epstein-Barr virus (EBV) have been investigated as possible causative agents of pterygium (2, 3).

Human herpesvirus 6 (HHV-6) belongs to the Roseolovirus genus of the Betaherpesvirinae subfamily and has a linear, double-stranded genome. The virus was first isolated in 1986 from AIDS patients with lymphoproliferative disorders (4). HHV-6 is an opportunistic pathogen with up to 90% seroprevalence in the human population and is commonly acquired during early childhood. The virus is separated into two major subgroups, HHV-6A and -6B, on the basis of genetic, immunological, epidemiological and functional features (4, 5).

Primary infection with HHV-6B can cause roseola (also known as sixth disease or exanthem subitum), a common childhood disease that resolves spontaneously. Although, HHV-6 infections are usually benign, the virus has been associated with several neurologic syndromes, such as febrile convulsions, encephalitis and multiple sclerosis. Besides roseola infantum, research on causative role of HHV-6 in other illnesses has been inconclusive (1, 6-8).

Overall, HHV-6 variant B is found more frequently than variant A in both peripheral blood mononuclear cells and solid tissues (5, 9). Variant A is predominantly present in the skin and active in nearly all Lyme disease patients. In some cases, both variants can coexist in the brain despite being localized in different brain areas (5, 9, 10). In addition, HHV-6 is reported to be associated with different cancers including Hodgkin’s lymphoma (HL), T-cell leukemia, EBV-negative B-cell lymphoma, African Burkitt’s lymphoma and adenomatous gastrointestinal polyps (6, 11-13). Given the association of HHV-6 with ocular diseases, we aimed to investigate presence of this virus in pterygium.

MATERIALS AND METHODS
With the aim to determine the prevalence of HHV-6 in specimens from patients with pterygium, this cross-sectional study was performed in the Department of Ophthalmology and Virology of Golestan University of Medical Sciences (Iran) between February 2013 and May 2015. The study received approval from ethics committee of the Golestan University of Medical Sciences. Fifty human biopsy specimens of pterygium were divided into three groups based on the grade of pterygium (grade 1: up to 2 mm, grade 2: 2-4 mm, and grade 3: more than 4 mm) (14). The samples were stored at -70 °C until processing.

DNA was extracted using QIAamp DNA Mini kits (Qiagen, Valencia, CA) according to the manufacturer’s instructions. The following β-globin primers were used to analyze quality of the extracted DNA after excluding false negative results; 5'-GAAGATGTTGATGGGATTTTCC-3' and 5'-GAAGGTGAAGGTCGGAGTC-3'. PCR was performed in a 25 μl reaction solution containing 5 μl of the extracted DNA (500 ng/ml), 0.1 μl of each primers with 10 pmol/μl, 0.3 U of Taq DNA polymerase (Genet Bio [A type], 0.5 μl dNTPs (10mM), 2 μl of MgCl₂ (25 mM) and 5 μl of 10X PCR reaction buffer. Amplification was done in a Peq Lab thermal cycler (Primus Advanced 96 thermal cycler) under the following conditions: 5 min at 95°C, 35 cycles of 30 s at 94°C, 30 s at 60°C, 30 s at 72°C and 72°C for 7 min.

Real-time PCR was performed in an ABI system 7300 with Maxima SYBR Green/ROX qPCR Master Mix (2X) (Ampliqon, Denmark) kit according to the manufacturer’s instructions (15). The primer pair 5'-TCGAAATAAGGCATTAGGCACACT-3' and 5'-CGGAGTTAAGGCATTGGTTGA-3' (98 bp) for the U22 open reading frame of HHV-6A and -6B was used in a 25-μL reaction mixture (containing 12.5 μL of PCR master mix) to evaluate the presence of HHV-6 sequences (15). Thermocycling conditions were as follows: 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s, 56 °C for 30 s and 60 °C for 30 s. An HHV-6-positive control was purchased from Vircell Microbiology Company (Figure 1). Finally, 98-bp amplicons were detected on 1% agarose gel containing 0.5 μg/ml ethidium bromide (Figure 2).
were postoperative recurrence. In the clinical examination for grading pterygia, 14 (28%) were atrophic/grade 1, 17 (34%) were intermediate/grade 2 and 19 (38%) were fleshy/grade 3. A positive family history of pterygium was noted in two cases (4%). Five positive cases (10%) had a history of outdoor activities. Seven patients (14%) had a positive smoking history. Six (12%) patients with pterygium were positive for HHV-6 DNA (Table 1).

PCR of the β-globin gene was used as an internal control for DNA extraction, which showed that all samples were β-globin positive. There was no statistically significant correlation between pterygium and the presence of HHV-6.

Prevalence of positive samples was calculated at 95% confidence interval (CI). Chi-square test and Fisher’s exact test were used to evaluate distribution of characteristics associated with HHV-6 infection. Statistical significance was set at 0.05 for statistical analyses. Demographic and clinical data were entered and analyzed with SPSS software (version 18).

RESULTS
Of 50 specimens examined in this study, 20 (40%) were collected from males and 30 (60%) were from females. Mean age of patients was 61.1±16.9 years (range: 22-85 years). Thirteen patients had bilateral pterygia, 42 specimen were primary lesions and eight were postoperative recurrence. In the clinical examination for grading pterygia, 14 (28%) were atrophic/grade 1, 17 (34%) were intermediate/grade 2 and 19 (38%) were fleshy/grade 3. A positive family history of pterygium was noted in two cases (4%). Five positive cases (10%) had a history of outdoor activities. Seven patients (14%) had a positive smoking history. Six (12%) patients with pterygium were positive for HHV-6 DNA (Table 1).

Table 1- Comparison of variables between patients with pterygium positive and negative for HHV-6 in Gorgan, Iran

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group</th>
<th>Positive for HHV-6 (n=6)</th>
<th>Negative for HHV-6 (n=44)</th>
<th>P-value (Chi-square test or Fisher’s exact test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>Male</td>
<td>2 (33.34%)</td>
<td>18 (40.9%)</td>
<td>0.252</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>4 (66.66%)</td>
<td>26 (50.09%)</td>
<td></td>
</tr>
<tr>
<td>Type of pterygium</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Primary</td>
<td>6 (100%)</td>
<td>36 (81.81%)</td>
<td>0.512</td>
</tr>
<tr>
<td></td>
<td>Recurrent</td>
<td>0</td>
<td>8 (18.18%)</td>
<td></td>
</tr>
<tr>
<td>Grade of pterygium</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Atrophic/grade1</td>
<td>4 (66.66%)</td>
<td>10 (22.72%)</td>
<td>0.253</td>
</tr>
<tr>
<td></td>
<td>Atrophic/grade2</td>
<td>0</td>
<td>17 (38.63%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Atrophic/grade3</td>
<td>2 (33.34%)</td>
<td>17 (38.63%)</td>
<td></td>
</tr>
<tr>
<td>Family history of pterygium</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>2 (33.33%)</td>
<td>0</td>
<td>0.816</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>4 (66.66%)</td>
<td>44 (100%)</td>
<td></td>
</tr>
<tr>
<td>History of outdoor activities</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>0</td>
<td>5 (11.36%)</td>
<td>0.435</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>6 (100%)</td>
<td>39 (88.63%)</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1-. (A) Dissociation curve. Violet: positive control, blue and pink: positive samples and green: negative control. (B) Amplification plot indicated Ct number in positive control, clinical samples (red line) and negative control (green line).

Figure 2-. Results of agarose gel electrophoresis of PCR products. C+: positive control, C-: negative control, M: DNA ladder (100 bp), lanes 1-3: HHV-6 positive specimens.
DISCUSSION

Many different genetic and environmental factors have been proposed to be involved in development of pterygium, but none has been found to be directly linked to the lesion formation.

The potential role of viral infections with HPV, EBV and herpes simplex virus as well as exogenous agents including ultraviolet irradiation in the development of pterygium remains controversial. However, since the disease creates a unique and favorable environment for viral oncoproteins, viruses might have a role in progression of the lesions (2).

Cell proliferation, interference with signaling pathways, resistance to apoptosis and inducing angiogenesis are the hallmarks of pterygium, all of which support the neoplastic nature of the lesion (16). However, pterygia are benign growths and typically do not require surgical excision until manifestation of acute symptoms, but recurrence is frequent after tissue excision (as high as 46%) (17).

Some studies have suggested an association between HHV-6 and ocular disease, corneal inflammation, optic neuropathy, tonic pupil, bilateral uveitis and Bell’s palsy. These results suggest that HHV-6 may be capable of invading the corneal tissue (6, 18-24).

Although HHV-6 is identified as an opportunistic virus, it has also shown transforming and trans-activating properties, thus playing a definitive role in human neoplasia (6, 9).

The detection of HHV-6 viral DNA sequences in some Hodgkin’s and non-Hodgkin’s lymphomas indicates the possible role of HHV-6 in human neoplasia (25,26).

The vast majority of studies utilized nested-PCR for diagnosing infection with HHV-6. Since pterygium usually grows slowly through life, even with this sensitive and specific technique, 30-60% of patients remain undiagnosed, because the HHV-6 may be chronically active in the tissue and only limited number of viruses circulate outside the tissue. Hence, real-time PCR must be sensitive-enough to detect HHV-6 infections in the tissues (24, 25, 27). In our study, the HHV-6 DNA was found in 6 (12%) patients using real-time PCR.

The limitation of this study were the small number of patients and not analyzing association between HHV-6 and variables such as grade of pterygium. To our knowledge, no study has investigated presence of HHV-6 in pterygium, but in a study by Crawford et al., 14 of 30 (46.7 %) central nervous system tumors were determined as HHV-6-positive using nested-PCR (27). A report in 2014 detected HHV-6 DNA in one of 21 (5%) cases of glioblastoma multiforme (28). Chi et al. reported that 17 of 40 (42.5%) glioma samples contained the HHV-6 DNA in nested-PCR (29).

Utilization of virus-specific laboratory assays including fluorescence in situ hybridization, loop-mediated isothermal amplification and immunohistochemistry could also be effective for clarifying virus replication in the lesion (6, 23, 24, 29). Recently, studies introduced a highly precise diagnostic tool named droplet digital–PCR, which has lower variability and outstanding quantification accuracy (26).

Our results suggest that HHV-6 infection may be just a bystander that creates an immunosuppressive milieu, which could contribute to disease severity. Elucidating whether the virus plays an oncogenic role in pterygium requires a more in-depth investigation.

CONCLUSION

Based on the results, a direct association between HHV-6 and development of pterygium seems less probable, which suggests that other etiologic agents must be involved in the multistep process of the disease.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.
REFERENCES


