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Investigation of human papillomavirus and Epstein-Barr virus DNAs in pterygium tissue

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PURPOSE. *Recent studies postulated the presence of a probable relationship between pterygium and neoplasia. This study aimed to investigate the role of two oncogenic viruses, human papillomavirus (HPV) and Epstein-Barr virus (EBV), in the development of conjunctival pterygia.*
METHODS. *Polymerase chain reaction was used to identify the presence of HPV and EBV in 30 primary and 10 recurrent pterygia samples. Twenty conjunctival samples obtained from patients undergoing cataract surgeries were used as the control group. Patient groups had similar sex, race, and age distribution to eliminate bias. For exploration of HPV in groups, two different PCR methods (in-house PCR with two different primer sets and one real-time PCR method) were studied. The presence of EBV was shown by real-time PCR method.*
RESULTS. *HPV was identified in none of the pterygia and control group patients. However, EBV was detected in 3 out of 30 (10%) primary pterygia patients and in none of the recurrent pterygia and control patients.*
CONCLUSIONS. *Up to now, HPV has been blamed as the major viral pathogen in the etiopathogenesis of pterygium. The current results suggest that EBV may also be involved in the pathogenesis of pterygium, but further larger studies with larger cohorts are required to confirm this hypothesis. (Eur J Ophthalmol 2009; 19: 175-9)*

KEY WORDS. *EBV, HPV, Pterygium, Real time PCR*

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INTRODUCTION

Pterygium is a condition characterized by the encroachment of a fleshy triangle of conjunctival tissue into the cornea. It is usually bilateral, and occasionally occupies nasal interpalpebral location. The pterygium epithelium that centripetally invades the cornea displays squamous metaplasia and goblet cell hyperplasia (1). Pterygium pathogenesis is considered to occur in two stages: disruption of the limbal corneal-conjunctival epithelial barrier and conjunctivalization of the cornea by tissue characterized by cellular proliferation, tissue remodeling, and angiogenesis (2).

Despite various studies, the pathogenesis of pterygium remains unclear. Chronic exposure to sun is the only factor of which the role has been clearly documented by epidemiologic (3, 4) and by in vitro (5) studies. The typical location of pterygium has been described by corneal focusing of the incident sunlight (2). Recent studies have provided data such as loss of heterozygosity and microsatellite instability, decreased apoptosis, increased growth factors, increased p53 expression, telomerase activity, and presence of oncogenic viruses, which support the concept that pterygia can be considered a neoplastic condition (6-10).
Some authors postulate a potential relationship between

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pterygia and neoplastic lesions and some studies presented the positivity of oncogenic viruses in the pterygia. In recent years, HPV have been the most commonly investigated virus in the etiopathogenesis of pterygia. However, HPV occurrence in pterygium lesion is controversial. In many studies, a positive correlation between pterygia and HPV infection has been reported (8, 11, 12), while others reported no such correlation (13, 14).

Epstein-Barr virus (EBV) is a member of the herpesvirus family and transmitted through oral secretions. EBV is another oncogenic virus, which predominantly affects blood cell lines and mucosal surfaces. Although its presence over ocular surfaces is well known, to our knowledge its presence in pterygia has not been shown.

The purpose of this study is to evaluate whether two oncogenic viruses, HPV and EBV, are involved in pterygium pathogenesis in our region.

METHODS

Patients with primary and recurrent pterygia without any other ocular pathology were included in the present study. Patients undergoing cataract surgery for age-related cataract were included in the study as the control group. None of the patients included had any immunosuppressive condition, or opportunistic infection. To be included in the study, informed consent was obtained from all participants. The study was approved by the ethical committee of Inonu University School of Medicine and carried out in accordance with the Declaration of Helsinki.

Samples

Between May 2006 and December 2007, 30 specimens of primary pterygia and 10 specimens of recurrent pterygia were collected. Also, 20 normal medial limbal conjunctiva biopsy specimens in 2x2 mm size were obtained from control patients.

For investigation of HPV in groups, two different PCR methods were used. These were real-time PCR (a PCR method designed as a ready-to-use commercial kit) and in-house PCR (a PCR performed by the reaction mixtures prepared manually in the laboratory). For in-house PCR detection kit, we used two different primer sets. For investigation of EBV in study groups, we used just a real-time PCR method.

Viral DNA isolation

Viral DNA was extracted from pterygia samples by the QIAcube automated extraction system with the QIAmp DNA Tissue Kit (Qiagen, Hilden, Germany). Two aliquots of the extracted DNA were made to avoid further freezing and thawing.

Qualitative HPV DNA detection by in-house PCR

For HPV detection and typing, a chip-based detection system (LCDarray HPV 3.5, Chipron GmbH, Germany) was used according to manufacturer's instructions. The specificity of the system was assessed for 32 HPV types (HPV 6, 11, 16, 18, 31, 33, 35, 39, 42, 44, 45, 51, 52, 53, 54, 56, 58, 59, 61, 62, 66, 67, 68, 70, 72, 73, 81, 82, 83, 84, 90, and 91). PCR was performed with two different primer sets provided with the assay for each sample. In brief, the biotinylated primer mix HPV MY09/11 and primer mix HPV 125 were used to generate labeled PCR products (~450 bp and ~125 bp length) from the L1 region of the HPV genome. An amount of 12 μL of prelabeled PCR products were mixed with the hybridization buffer and hybridized to the arrays for 30 minutes at 35 °C. Following a short washing step, the arrays were incubated with the secondary label solution for 5 minutes at room temperature and washed again. Hybridization events were visualized by means of the staining solution. Within 1 to 2 minutes, the substrate formed a dark blue precipitate in those positions where PCR amplicons were bound. Following the staining procedure, high resolution images were taken with a transmission light-scanning device and automated data analysis was done with the "SlideReader" software (LCD-Analysis package, Chipron, Germany).

Quantitative HPV DNA detection by real-time PCR

Real-time PCR was performed on the LightCycler 2.0 instrument (Roche Diagnostics Corporation, Indianapolis, IN). All samples were tested with the molecular beacon-based one-step multiplex real-time PCR system (GenoID Real-Time HPV Assay, Budapest, Hungary) for identification of certain high-risk (HPV types 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) and low-risk HPV types (HPV types 6, 11, 42, 43, and 44/45). 5′-FAM-3′-

DABCYL-labeled molecular beacons were used for detecting the high-risk types while detection of the low-risk types was done by 5′-TET-3′-DABCYL-labeled beacons. The internal control which was added before sample DNA extraction was detected by a 5′-FAM-TexasRed-3′-DABCYL wavelength-shifting molecular beacon. For the detection of high-risk types, low-risk types, and internal control, 530, 560, and 610 nm fluorescence were used, respectively. The detection limits of the assay were between 22 and 700 copies/reaction (15).

Quantitative EBV DNA detection by real-time PCR

The EBV viral load in the pterygia samples was determined by quantitative real-time PCR on a RG-3000 cycler (Corbett Research, Wasserburg am Inn, Germany) using the RealArt™ EBV RG PCR kit (Artus, Hamburg, Germany). The detection limit of the assay, according to the manufacturer, was 3.8 copies/μL (p=0.05) in the case of EBV. An internal control DNA (included in the kit) was added to each sample prior to extraction to ensure that there was no DNA loss during extraction and there were no inhibitory substances in the extract as an independent PCR based on the internal control DNA was done for each sample.

RESULTS

The demographic features and viral quantities of patients are shown in Table I. We did not detect any HPV DNA fragments from either pterygia groups or from control group. On the other hand, we detected EBV DNA in 3 out of 30 (10%) primary pterygia specimens; however, none of the recurrent pterygia or normal conjunctiva specimens contained EBV DNA.

DISCUSSION

The oncogenic potential of HPV and its potential contribution to development of various preneoplastic and neoplastic conditions has been proved. DNA of HPV, particularly of types 16 and 18, has been detected in various forms of neoplastic conditions, observed on the eyelids, conjunctiva, and cornea (16, 17).

The close relation between HPV and conjunctival epithelial pathologies ranging from papillomas to squamous cell carcinoma has been previously documented (18). As mentioned previously, there are conflicting results about the relation between HPV and pterygia. Some studies mentioned a strong correlation between HPV and pterygia (8, 11, 12) while, in contrast, there are also studies which are in concordance with our results (13, 14).

A possible explanation for the controversial results of different studies and lack of positive correlation in our study may be the variation in the geographic region. The incidence of HPV in pterygia was 50% in patients from the United Kingdom (12), 100% in patients from Italy, but 21% among Ecuadorian patients (11). In Brazilian patients and in Chinese patients from Taiwan, the HPV in pterygium samples was 100% negative (13, 14).

There are also controversial results with respect to the HPV types which were detected in pterygia. The family *Papillomaviridae* consists of more than 180 distinct types of viruses, 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 (19). Although HPV 16 and 18 are the main types detected in conjunctival neoplasms and even in healthy conjunctiva (18), investigations have shown that, in addition to these two strains, types 6 and 11 have also been detected in pterygia (8, 12). There are also reports of different HPV types from different study groups. Piras et al detected three unusual strains of HPV in pterygia, named type 52, 54, and 90 (11). The importance of the study was that the detected types were the same in

TABLE I - DEMOGRAPHIC FEATURES AND QUANTITY OF VIRUS IN PATIENTS WITH PRIMARY PTERYGIA, RECURRENT PTERYGIA, AND CONTROLS

Sample	EBV	HPV
Primary pterygia (n=30)	3 (10%)	0
Range of viral copies	100–268	
Gender (F/M)	12/18	
Mean age ± SD	63.8±12.1	
Recurrent pterygia (n=10)	0	0
Range of viral copies		
Gender (F/M)	3/7	
Mean age ± SD	64.8±16.0	
Control (n=20)	0	0
Range of viral copies		
Gender (F/M)	10/10	
Mean age ± SD	66.0±7.8	

specimens from different geographic regions.
The technique used could be another reason for highly different results from different study groups. Sjö et al detected HPV type 6 DNA in 4 of 90 suitable pterygium by PCR but the authors could not confirm the results with DNA in situ hybridization (20). Chen et al investigated the samples with three different PCR primers however they could not find HPV/DNA in their study group. Thus they proposed that HPV is not a cofactor in etiopathogenesis of pterygia (14). Although we could not detect HPV in any of our results, from our country Varinli et al reported the presence of HPV in pterygia (21). This discrepancy may be related to geographic difference and also may be related to the methods used for investigation of HPV.
EBV is a member of the herpesvirus family and transmitted through oral secretions. It is the most common etiologic agent of infectious mononucleosis (IM). It has also been implicated in nasopharyngeal carcinoma, Sjögren syndrome, oral hairy leukoplakia, and Burkitt lymphoma. Ocular findings in IM encompass a wide range of clinical signs affecting both neuro-ophthalmologic and anterior segments. Diseases of the anterior segment include follicular conjunctivitis, dendritic epithelial keratitis, and stromal keratitis (22). Recently, Restelli et al demonstrated the presence of EBV nuclear antigen-1 (EBNA-1) by immunohistochemistry in 4 of 15 cases of conjunctival squamous carcinomas and related dysplasias (23). In addition, the presence of EBV has been convincingly demonstrated in lymphocytic infiltrations of conjunctiva (24, 25). However, to our knowledge, there have been no reports linking EBV with pterygium.
The real-time PCR technique that we used has high sensitivity for detection of low virus load. On the other hand, it has been postulated that the nucleic acid quality within paraffin fixed tissue specimens is highly dependent on a large number of parameters that can lead to the degradation of nucleic acids (26). Based on this knowledge, we preferred to use fresh frozen pterygia tissue in this study as this is a prospective study in which the fresh frozen tissues were available during the study period. There are no reports in the literature evaluating EBV in either fresh frozen tissue or paraffinized tissue; thus it is not possible to make a comparison between outcomes.
The results of this study showed that in the etiopathogenesis of pterygia, different viruses may participate in different geographic regions. However, elucidation of this hypothesis needs organization of a multicentric study in which tissues are investigated in a central laboratory.

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