HHS Public Access

Author manuscript

Exp Eye Res. Author manuscript; available in PMC 2021 December 07.

Published in final edited form as:

Exp Eye Res. 2020 October ; 199: 108194. doi:10.1016/j.exer.2020.108194.

Relative gene expression analysis of human pterygium tissues and UV radiation-evoked gene expression patterns in corneal and conjunctival cells

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Abstract

A sight threatening, pterygium is a common ocular surface disorders identified by fibrovascular growth of the cornea and induced by variety of stress factors, like ultraviolet (UV) exposure. However, the genes involved in the etiopathogenesis of this disease is not well studied. Herein, we identified the gene expression pattern of pterygium and examined the expression of pterygium-related genes in UV-B-induced human primary cultured corneal epithelial cells (HCEpCs), telomerase immortalized human corneal epithelial (hTCEpi), primary conjunctival fibroblast (HConFs) and primary pterygium fibroblast cells (HPFCs). A careful analysis revealed that the expression of 10 genes was significantly modulated (by > 10-fold). Keratin 24 (*KRT24*) and matrix metalloproteinase 9 (*MMP-9*) were dramatically upregulated by 49.446- and 24.214-fold, respectively. Intriguingly, UV-B exposure (50 J/m^2) induced the upregulation of the expressions of *MMP-9* in corneal epithelial cells such as HCEpCs and hTCEpi. Furthermore, UV-B exposure (100 and/or 200 J/m^2) induced the upregulation of the expressions of *MMP-9* in fibroblast such as HConFs and HPFCs. The exposure of HCEpCs to 100 and 200 J/m^2 UV-B induced significant expressions of *KRT2*4 mRNA. Nevertheless, no expression of *KRT2*4 mRNA was detected in HConFs and HPFCs. The findings provide evidence that the progression of pterygium may involve the modulation of extracellular matrix-related genes and vasculature development and the up-regulation of *KRT24* and *MMP*-9 by UV stress. UV radiation may promote the modulation of these pterygium-related genes and induce the initiation and progression of human pterygium.

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Declaration of competing interest

All authors have no conflicts of interest to disclose relating to this work.

Keywords

Pterygium; MMP-9; KRT24; Corneal epithelial cells; Conjunctival fibroblast; UV-B

1. Introduction

Eye is maximally exposed to environmental stressors like ultraviolet (UV) radiation, which eventually affects cellular integrity of cells by aberrantly regulating gene expression and function. Recent compelling evidence revealed that UV radiation is clinical risk factor for progression of pterygium. A pterygium is an epithelial and fibrovascular proliferation of the conjunctiva (Cameron, 1983; Coroneo et al., 1999). If not retarded, an advancing pterygium generates significant changes in the refractive index and corneal curvature (Bedrossian, 1960; Tomidokoro et al., 2000) and its progression would subsequently invade the cornea, forming a wing-like shape and causing visual loss. It is estimated that more than 200million people is affected due to pterygium (Lucas et al., 2008). Nonetheless, effective treatment for pterygium has not yet been identified, and surgical removal is the only way to remove a pterygium at present (Gris et al., 2000). Hence, it is highly imperative to identify the factors (genes) contributing to pterygium etiology and thereby develop gene targeted therapy.

Several studies have shown that pterygium formation is stimulated by various factors, including UV radiation (Zhou et al., 2016). UV exposure of pterygium epithelial cells generates proinflammatory cytokines, such as IL6 and IL8 (Di Girolamo et al., 2006). In addition, UV-B-driven oxidative stress can induce other inflammatory factors, like IL1α and TNFα that can lead other delirious signaling by activating MMPs Also, the progression of a pterygium may be the result of excessive wound healing, cell proliferation, and a limbal stem-cell deficiency triggered by oxidative stress due to UV exposure, and/or an inflammatory mediator, immunologic mechanisms, growth factors, or viruses on the ocular surface (Coroneo et al., 1999; Di Girolamo et al., 2004; Jaworski et al., 2009; Threlfall and English, 1999). The parts of pterygium are head (apical part present in cornea), neck (limbal part) and body (scleral part). There is evidence that UV-mediated limbal damage accelerates pterygium pathogenesis, and this has been reviewed extensively by Coroneo and colleagues (Coroneo, 1993; Coroneo et al., 1999; Di Girolamo et al., 2004). Furthermore, UV radiation is known to cause oxidative DNA damage, and proposed to be a major event in the pathogenesis of pterygium. From the above reports, it appears that pterygium formation is multifactorial, however, the mechanisms underlying the development of pterygium are not fully known (Coroneo, 1993; Threlfall and English, 1999).

In the context of primary pterygium, there are various reports on oxidative stress and the antioxidant defense balance (Balci et al., 2011; Kormanovski et al., 2014). Enhanced levels of nitric oxide and reduced levels antioxidants, such as superoxide dismutase and catalase have been shown in primary pterygium (Balci et al., 2011; Kormanovski et al., 2014). The marker 8-hydroxydeoxyguanosine, a sensitive and stable marker commonly used to identify oxidative damage to DNA, has been observed in pterygium in some studies (Kau et al., 2006; Maxia et al., 2008; Tsai et al., 2005), dictating the a major role of oxidative stress in pterygium pathobiology.

Moreover, the progression of a pterygium is signalized by the degradation of the corneal basement membrane and extracellular matrix (ECM) and a proliferation of fibrovascular tissue (Cameron, 1983; Coroneo et al., 1999; Di Girolamo et al., 2004). It has been observed that fibroblasts from pterygium have characteristics of myofibroblasts (Dushku and Reid, 1994; Kato et al., 2007). Also, the expression of several types of matrix metalloproteinases (MMPs) (MMP-1, 2, 3, 7, and 9) increases in pterygium (Chao et al., 2011; John-Aryankalayil et al., 2006; Yang et al., 2009). It has been demonstrated that MMP-2 and -9 are overexpressed in pterygium tissue and fibroblasts isolated from pterygium (Yang et al., 2009), suggesting that the levels of these MMPs in pterygium and its fibroblasts may be linked to the progression of pterygium.

Herein, by employing more extensive, microarray study, we identified relative gene expression profiles that are modulated in apical portion of pterygium compared to normal uninvolved conjunctival tissues. In addition, we compared the gene expressions between apical and basal portion of pterygium. Specifically, we also investigated the effect of UV-B exposure on the expression of *MMP-9* proposed to play role in pterygium progression, using human pterygium tissues. The outcomes of the study can pave the way to develop therapeutic agents for the treatment/prevention of pterygium based upon target gene(s).

2. Materials and methods

2.1. Human pterygium sampling

This study was approved by the institutional review board of Kanazawa Medical University (Approval code: 78) with the appropriate informed consent obtained from all patients. Fifteen pterygium specimens were collected from 12 patients aged 70.539 ± 11.163 (mean ± standard deviation: SD) years and as controls, conjunctival tissues from conjunctivochalasis (Con) were obtained from four patients aged 79.750 ± 3.775 years at Kanazawa Medical University Hospital. There was no significant difference in age between the two groups. Three samples taken from the right eye of three patients were used for the microarray, and three samples taken from the left eye were used for the reverse transcribed quantitive real-time polymerase chain reaction (RT-qPCR) method. In addition, 9 samples collected from 9 patients were used for the RT-qPCR method in addition to the above 3 samples. As the normal controls, it was impossible to collect the normal conjunctiva near the limbus from the patient because the consent of the patient could not be obtained. Further, maintaining the normal conjunctiva is important in the treatment of other eye diseases in the future, and therefore excision of the normal conjunctiva from human patients is difficult. Due to these reasons, control conjunctival samples were obtained from conjunctivochalasis specimens which were excised by making a crescentic excision of the loose inferior bulbar conjunctiva starting with a peritomy approximately 3 mm posterior to the limbus including the conjunctival laxative part exposed from the eyelid margin.

Excised pterygium tissues were divided into apical portion including head (apical part present in cornea) and neck (limbal part) (PA) and basal portions (PB). PA and PB tissues were further divided laterally into two identical and symmetric pieces (Fig. 1). One piece was stored in RNAlater solution (Ambion®, ThermoFisher Scientific Japan, Tokyo) to stabilize the RNA until processing for a microarray or RT-qPCR. The other piece was frozen

and used immediately for the isolation of protein as described as below or fixed with 10% Formalin Neutral Buffer Solution (Wako, Osaka, Japan) for immunohistochemistry.

2.2. Cell culture and UV-B treatment

Primary human corneal epithelial cells (HCEpCs) were obtained from Cell Applications (San Diego, CA, USA). Human telomerase-immortalized corneal epithelial cell line (hTCEpi) was obtained from Evercyte GmbH (Vienna, Austria). HCEpCs and hTCEpi were cultured with KGM™-2 Keratinocyte Growth Medium-2 (Lonza Japan Ltd, Osaka, Japan) at 37 °C in an air-CO_2 (19:1) atmosphere. Primary human conjunctival fibroblasts (HConFs) were obtained from ScienCell Research Laboratories (Carlsbad, CA) and cultured with Fibroblast Medium (ScienCell) at 37 °C in an air-CO_2 (19:1) atmosphere. Primary pterygium fibroblast cells (HPFCs) were established by ourselves from human pterygium specimens by using a technique previously reported (Li et al., 2001). HPFCs were subsequently spread in 75-cm^2 culture flasks (Nunc, Roskilde, Denmark) in with Fibroblast Medium (ScienCell) and 100 U/ml penicillin and 100 μg/mL streptomycin (ThermoFisher) at 37 °C in an air-CO_2 (19:1) atmosphere. First, 1×10^5 of HCEpCs, HConFs, hTCEpis and HPFCs were seeded on 35-mm culture dishes at 37 °C in an air-CO_2 (19:1) atmosphere for 24 h.

For the UV-B irradiation assays (Fig. 2A), the HConFs, HCEpCs and HPFs were placed in 1 mL of 1X phosphate-buffered saline (PBS), and irradiated with 0, 50, 100, or 200 J/m^2 UV-B once per day for 2 and/or 4 days,. The medium was then changed to fresh medium. Ultraviolet-B light was generated by a 15-W UV-B light source (312 nm), with its intensity standardized using a UV light meter (UVP, Upland, CA). hTCEpis were placed in 1 mL of 1X PBS, and irradiated with 0, 50 or 100 J/m^2 UV-B once per day for 2 days (Fig. 2B). Experiments were repeated three times for each cell type.

2.3. RNA extraction

Total RNA from each human sample and cultured HConFs, HCEpCs, HPFC and hTCEpi cells was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA) by following the manufacturer's protocol. Samples of RNA were set aside for a RT-qPCR to verify the results obtained from the microarray analysis.

2.4. Microarray analysis and gene ontology analysis

Pterygium-related genes were screened by microarray analysis using pterygium samples as follows. Three apical portions of pterygium specimens from primary patients (PA-1, PA-2, PA-3) and one control conjunctival tissue (Con-1) were used for the microarray analysis, and all four samples were processed for the microarray analysis as follows. We used the Agilent Low Input Quick Amp Labeling Kit (Agilent Technologies, Santa Clara, CA) for RNA labeling. Briefly, for each labeling, 100 ng of total RNA was reverse-transcribed in the presence of CDS primer mix and MMLV reverse transcriptase. Cyanine 3-CTP was coupled to the first-strand cDNA. Labeled cDNA was hybridized to SurePrint G3 Human 8 × 60K microarray kit v2 (Agilent) using the Agilent® Gene Expression Hybridization Kit according to the manufacturer's protocol. Air-dried slides were scanned with an Agilent

Microarray Scanner. Scanned images were analyzed with Feature Extraction Software (v. 10.7.3.1) (Agilent).

Per chip normalization was done by dividing each gene's measurement by the specific control measurements or by the average intensity in the single array. Normalized data were exported for the subsequent analysis. Genes with a normalized ratio >2.0-fold or <0.5-fold between three samples were selected as significant genes for the progression of pterygium.

2.5. RT-qPCR

To measure the expression of mouse and human *MMP-9* and *KRT2*4 mRNAs, we conducted a relative quantification of mRNA using a Prism7300 (Applied Biosystems, ThermoFisher Scientific Japan). The comparative Ct method was used for the relative quantification of miRNA expression. The PCR amplification was performed using TaqMan Universal Master Mix and pre-developed human *MMP-9* and *KRT24* probe mix (Applied Biosystems). The relative quantity of each mRNA was determined using the comparative Ct method and then normalized using a pre-developed TaqMan ribosomal RNA control reagent VIC probe as an endogenous control (Applied Biosystems).

2.6. Statistical analysis

For all quantitative data collected, the statistical analysis was conducted with Turkey test or Student's t-test when appropriate. The data are presented as the mean ± SD of the indicated number of experiments. A significant difference between the control and treatment group was defined as a p-value <0.05 for two or more independent experiments.

3. Results

3.1. Gene expression profiling and analysis of pterygium

As described above, three apical portions of pterygium specimens from primary patients (PA-1, PA-2, PA-3) and one control conjunctival tissue (Cont-1) were used for the microarray analysis. The data for the microarray analysis was deposited to Gene expression omnibus (GEO) database (Accession number: GSE151872). There were 957 genes in the PA-1 group, 1110 genes in the PA-2 group, and 1194 genes in the PA-3 group that showed significant changes of greater than 2.0-fold. Table 1 provides the list of the 8 top-ranked genes that showed significant changes (>2.0-fold) in each three pterygium patients (PA-1-3). Table 2 provides the list of the 30 top-ranked genes that showed significant changes (>2.0-fold) that were common to all three pterygium patients (PA-1-3).

The most highly up-regulated gene family that was detected in all three samples was *KRT24*, the superfamily of intermediate filament proteins, which showed a >49.446-fold higher expression compared to the control. *MMP-9*, which belongs to a class of enzymes that is involved in the degradation of ECM, was up-regulated by more than 24.214-fold. Our gene ontology analyses revealed an upregulation of a set of genes classified in ECM, extracellular regions, regulation of cell migration, and vasculature development in all three pterygium samples (Table 3).

Significant changes that were <0.5-fold were detected in 897 genes in the PA-1 specimens, 1516 genes in the PA-2 specimens, and 1785 genes in the PA-3 specimens. Table 4 is the list of the 30 top-ranked genes that showed significant changes <0.5-fold in common to all groups. Gene ontology analyses revealed a down-regulation of genes related to the defense response to viruses, the cellular response to type I interferon, the type I interferon signaling pathway, and the response to type I interferon in two of the three pterygium patients' samples (Table 5).

3.2. Validation of the expression of KRT24 and MMP-9 mRNA modulated in microarray

The results of the microarray analysis presented above showed the modulation in the expression levels of several genes during the progression of pterygium, indicating the importance of these genes' regulatory roles in the gene expression during pterygium development. However, we observed that genes belonging to the same class/family had similar expression profiles, suggesting that they may not be differentiated in a microarray analysis due to the possibility of cross-hybridization. To avoid this, we selected *KRT24* and *MMP-9*, whose expression was modulated in our microarray data, and we validated their expression by real-time RT-qPCR (Fig. 3).

We analyzed samples of three normal conjunctiva of conjunctivochalasis as control samples (Con) and 12 pterygium samples for the real time RT-qPCR. *KRT24* (A) and *MMP-9* (B) mRNA were dramatically altered in both the apical and basal portions of pterygium (Fig. 3A and B). The expression of *KRT2*4 mRNA in the basal (PB) and apical portions (PA) of pterygium was significantly up-regulated in comparison to the expression in the control samples (Con) (Fig. 3A, *$p < 0.05$ and **$p < 0.025$ vs. control). The expression of *MMP*-9 mRNA in the basal and apical portions of the pterygium samples was significantly up-regulated compared to the control samples (Fig. 3B, *$p < 0.025$vs. control).

3.3. UV-B-induced expression of KRT24 and/or MMP-9 mRNA in HCEpC, HConF, hTCEpi and HPFC

To determine whether the expressions of *KRT24* and/or *MMP*-9 mRNA are up-regulated in cells exposed to UV-B which is proposed to be a causative factor of pterygium, we exposed HCEpC and HConF cells exposed to 0, 50, 100, or 200 J/m^2 UV-B once a day for 4 days, and total RNA extracts were prepared on Day 2 and/or Day 4 from each. We then performed an RT-qPCR (n = 3 in each group). The exposure of HCEpC cells to 100 and 200 J/m^2 UV-B also induced significant expressions of *KRT2*4 mRNA on Day 4 (Fig. 4A; *$p < 0.02$, 0 vs 200 J/m^2; **$p < 0.04$, 50 vs 200 J/m^2). The exposure of HCEpC cells to 50 J/m^2 UV-B significantly induced the expression of *MMP-9* on Day 2 and 4 (Fig. 4B; *$p < 0.007$, ***$p < 0.001$ vs. 0 J/m^2). On Day2 and 4, the expression of *MMP-9* was significantly decreased after the exposure of 100 and 200 J/m^2 (Fig. 4B; *$p < 0.007$, **$p < 0.05$, ***$p < 0.001$ vs. 50 J/m^2). UV-B In addition, the exposure of HConF cells to 50 or 200 J/m^2 UV-B induced a significant expression of *MMP*-9 mRNA on Day 4 (*$p < 0.001$, **$p < 0.03$ vs. 0 J/m^2) (Fig. 5A). In compared toHConF cells after exposure of UV-B for 4 days, the exposure of HConF cells to 100 J/m^2 UV-B induced a significant up-regulation of *MMP*-9 mRNA (***$p < 0.005$) (Fig. 5A). The exposure of HPF cells to 100 and 200 J/m^2 UV-B significantly induced the expression of *MMP-9* on Day2 (Fig. 5B; *$p < 0.05$ vs. 200 J/m^2). The exposure

of hTCEpi cells to 50 J/m^2 UV-B significantly induced the expression of *MMP-9* on Day 2 (Fig. 5C; *p < 0.025 vs. 0 and 100 J/m^2). No expression of *KRT24* mRNA was detected in HConF cells and HPF cells. There was no significant change in expression of *KRT24* after UV-B exposure to hTCEpi cells, because expression of *KRT24* was very low level in hTCEpi cells (Data not shown). Our analyses demonstrated that *KRT24* is specifically expressed in HCEpCs.

4. Discussion

A gene expression analysis of pterygium demonstrated that several pathways were significantly affected. The analysis revealed that the tissue-specific markers, *KRT24* and *MMP-9* were the most abundant in the apical portion of the pterygium. *KRT24* is a member of the type I (acidic) keratin family, which belongs to the superfamily of intermediate filament proteins (Schweizer et al., 2006) that was identified in Naegeli-Franceschetti-Jadassohn syndrome and dermatopathia pigmentosa reticularis (Sprecher et al., 2002). *KRT24* influences the cellular response to proapoptotic signals and the routing of membrane proteins in polarized epithelial cells (Hong et al., 2007). Further, *KRT24* was consistently up-regulated in the mucosa of colorectal cancer patients (Hong et al., 2007). *KRT24* has also been reported as a terminally differentiated gene for corneal stromal cells (Hashmani et al., 2013).

In our present study, *KRT24* was highly up-regulated in the apical portion of pterygium specimens in both the microarray analysis and RT-q PCR compared to the conjunctival control tissues and basal portion of pterygium tissue. In compared to control conjunctiva, the expression of *KRT24* was also significantly increased in the basal portion of the pterygium, which may have not included corneal epithelial cells. Recently, it was found that *KRT24* was up-regulated in pterygium using microarray analysis compared to its expression in tissues from conjunctiva, but it could not confirm by RT-qPCR analysis (Zhang and Liu, 2019). Furthermore, Keratin K24, which is encoded by the *KRT24* gene that is located at one end of the type I keratin gene cluster, is highly expressed in the superficial layer of the corneal epithelium point to a predominant role of human K24 in the cornea (Ehrlich et al., 2019). The expression of *KRT24* was not detected in the HConF cells, and its expression in the control conjunctival tissues was low-level. We speculate that *KRT24* is related to the transformation of limbal tissues to pterygium and that *KRT24* may be a marker of pterygium.

Moreover, based upon accumulating evidence, we think that UV-B induces the expressions of genes that may contribute to the progression of pterygium. Toward this, cells were exposed to UV-B as described in ‘Materials and Methods’ section. In cultured HCEpC cells, we found, for the first time that expression of *KRT24* was induced after UV-B exposure in HCEpC cells (Fig. 4A). Pterygium is a condition of the ocular surface characterized by squamous cell metaplasia and goblet cell hyperplasia. We believe that because UV-B is one of the causative factors of pterygium, UV-B-driven aberrant *KRT24* expression could induce the epithelial transformation of limbal stem cells, resulting in the pathogenesis of pterygium.

Our findings regarding the upregulation of *MMP-9* are consistent with those of previous studies. We also observed that *MMP*-9 mRNA was highly upregulated in both apical and basal portions of pterygium. MMP-9 (gelatinase B, 92-kDa gelatinase, 92-kDa type IV collagenase) is a member of the MMP family, which plays a role in the proteolysis of ECM (Kahari and Saarialho-Kere, 1999; Yang et al., 2009). Gelatinases are able to perform the final degradation of fibrillar collagens after their first cleavage by collagenases (Kahari and Saarialho-Kere, 1999). The expression of various MMPs is increased in epithelial cells and fibroblasts in pterygium (Dushku et al., 2001; Seet et al., 2012; Yang et al., 2009). An increased expression of MMPs dissolves Bowman's layer in the cornea and induces angiogenesis and the migration and proliferation of pterygium onto the cornea (Yang et al., 2009). In addition, MMP-9 can degrade cell matrix proteins such as fibronectin, laminin, elastin, and various collagens (types I, II, and V) (Kahari and Saarialho-Kere, 1999; Okada et al., 1995).

Furthermore, our work revealed that UV-B increased the expression of *MMP*-9 mRNA in HCEpC, hTCEpi, HConF and HPFCs. In hCEpCs and hTCEpi, the expression of *MMP9* was increased only by UV-B irradiation at 50 J/m^2. In corneal epithelial cells, *MMP9* may be induced only by mild UV-B irradiation and may be involved in the early pterygium development and degradation of corneal epithelium. Moreover, when the intensity of UV-B irradiation is increased (>100 J/m^2), the expression of *MMP9* in fibroblasts such as HConFs and HPFs was increased. In conjunctival fibroblast, stronger intensity of UV-B irradiation (>100 J/m^2) may be needed for MMP9 induction to induce the pathogenesis of pterygium. In an earlier study, it was observed that the expression of *MMP-1* was induced by UV-B exposure (Di Girolamo et al., 2003). Collectively, the significant inductions of *MMP-9* and *KRT2*4 mRNAs after UV-B exposure suggest a plausible role of UV-B in the pathophysiology of pterygia. Our gene ontology analysis revealed the genes that may be related to the progression of pterygium; for example, ECM-related genes and genes related to vasculature development and the regulation of cell migration were significantly increased. Pterygia are highly vascularized, proliferative, degenerative and invasive ocular surface lesions that originate at the corneal limbus (Di Girolamo et al., 2003; Dushku and Reid, 1994). The genes that we observed in the microarray analysis may be involved in the progression of pterygia and provide a clue to develop specific transcription based therapy for treating pterygium, in future.

Moreover, several research groups have shown that altered corneal limbal epithelial cells undergo epithelial-mesenchymal transition (EMT) influenced by the Wnt/β-catenin pathway and microRNA (miRNA)-200 (Ando et al., 2011; Kato et al., 2007; Kim et al., 2016). In addition, the fibrovascular change is more severe in recurrent pterygia than at the initial site of presentation (Tan et al., 1997; Touhami et al., 2005). It was reported that myofibroblasts with tumor-inducing phenotypes express α smooth muscle actin (αSMA) and that αSMA was also expressed in pterygia (Kato et al., 2007; Touhami et al., 2005). Pterygium has also been described as a benign neoplastic lesion (Dushku et al., 2001; Tan et al., 2000; Weinstein et al., 2002). Thus, pterygia may have tumor-like characteristics. We surmised that since myofibroblastic changes in pterygia are observed in the basal portion, the EMT may be induced in the basal portion of conjunctival tissues, leading to the progression of pterygia.

Our analyses also demonstrated that defensins were highly down-regulated in the pterygium tissues. The gene ontology analysis showed the genes that function in the defense response to viruses and the response to type I interferon. Defensins are cationic antimicrobial peptides characterized by the presence of six cysteine residues linked to form three disulfide bridges. Two forms of human defensin, α and β, are recognized, depending on the location and connectivity of the cysteines. β-defensins are expressed by many epithelia including the cornea (Ganz and Lehrer, 1995; Haynes et al., 1999; McDermott et al., 2003). Six human β-defensins (hBD-1 through –6) have been identified to date (Bensch et al., 1995; Garcia et al., 2001a, 2001b; McDermott et al., 2003; Tomita et al., 2002; Yamaguchi et al., 2002). hBD-1 is constitutively expressed, whereas hBD-2 and -3 are inducible by cytokines and bacterial products. hBD-4 appears to have a more limited distribution than hBD-1,- 2, or -3 (Garcia et al., 2001a, 2001b).

Defensins have a broad spectrum of antimicrobial activity, being effective against many Gram-positive and -negative bacteria, some fungi, and enveloped viruses (Ganz and Lehrer, 1995). Human papillomavirus (HPV) infection has been reported as a possible inducing factor of pterygium. A closer look of the literature indicated that the prevalence of HPV in ocular surface diseases varies over a wide range (0%–100%) (Di Girolamo, 2012; Woods et al., 2013). This variance may be linked to the many different types of assays applied to detect the virus, as well as geography and genetic susceptibility (Di Girolamo, 2012; Woods et al., 2013). The existing literature indicated that approx. 60% of the published studies identified HPV in pterygia tissues, with an overall prevalence of 19% (Di Girolamo, 2012; Woods et al., 2013). The decreased defense response against a virus in limbal tissues may allow the infection of HPV or other viruses or bacteria and may induce the progression of a pterygium.

5. Conclusion

In conclusion, our study provides the evidence the involvement of UV-B and UV-B-driven aberrant expression of *MMPs*, and *KRT24* in the development of pterygia. The finding may help in the identification of new therapeutic target and the design of new approaches for the treatment and prevention of pterygia.

Funding

This work was supported by grants from Japan Society for the Promotion of Science (JSPS) KAKENHI Grant Numbers JP 17K16991 (to NK), a Grant for Promoted Research from Kanazawa Medical University [S2016-9] (to NK) and National EYE Institute, National Institute of Health (NIH) (EY024589) to (DPS).

Abbreviations:

UV	ultraviolet
HCEpCs	human primary cultured corneal epithelial cells
hTCEpi	telomerase immortalized human corneal epithelial
HConFs	primary conjunctival fibroblast

HPFCs	primary pterygium fibroblast cells
KRT24	Keratin 24
MMP-9	matrix metalloproteinase 9
ROS	reactive oxygen species
ECM	extracellular matrix
SD	standard deviation
Con	conjunctivochalasis
PA	apical portion
PB	basal portions
RT-qPCR	reverse transcribed quantitive real-time polymerase chain reaction
PBS	phosphate-buffered saline
ANOVA	one-way analysis of variance
EMT	epithelial-mesenchymal transition
miRNA	micro RNA
αSMA	α smooth muscle actin
hBD	β-defensins
HPV	human papillomavirus

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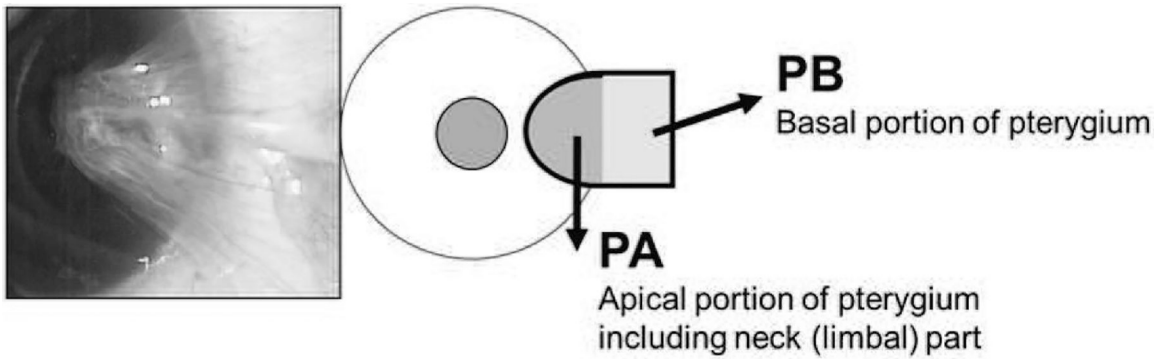

Fig. 1.

Method of pterygium sample collection. Human pterygium tissues were surgically exciced and divided in apical portion including head and neck (limbal part) (PA) and basal portion (PB).

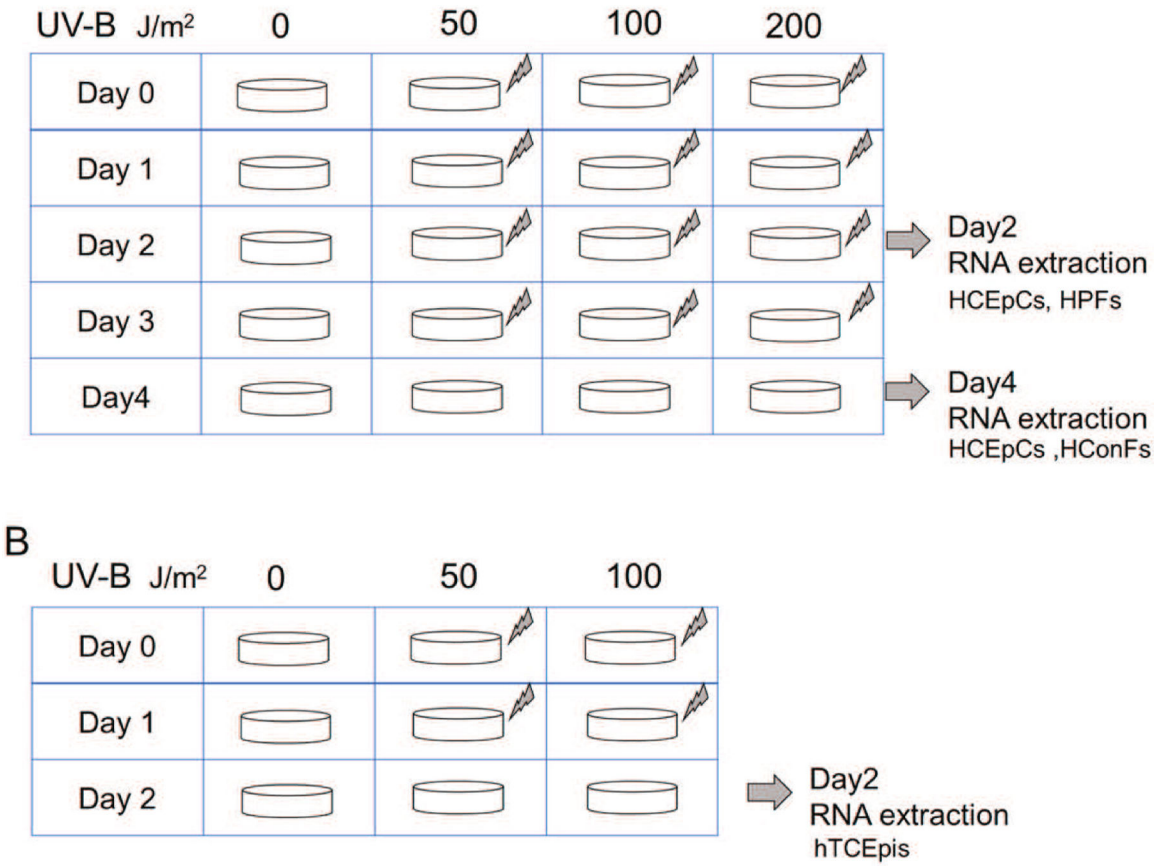

Fig. 2.

Illustration of method for culture experiments. UV-B was irradiated with varying intensity (A: 0, 50, 100, 200 J/m^2) or (B: 0, 50, 100 J/m^2) every day, and RNA extraction was performed on the second and/or fourth day.

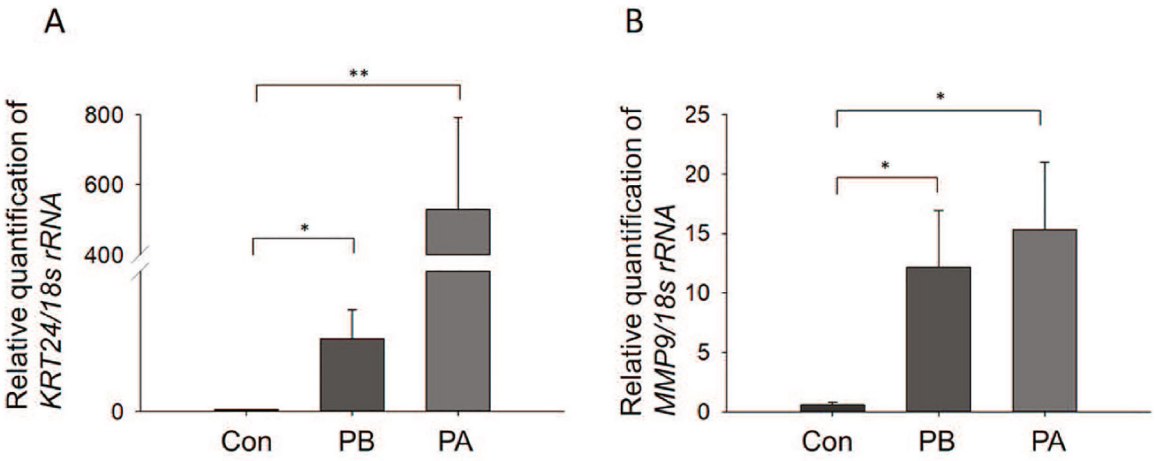

Fig. 3.

Expression of *MMP-9* and *KRT2*4 mRNAs in human pterygium samples. (A) Comparison of *MMP-*9 mRNA levels between three control conjunctiva tissues and 12 pterygium tissues at the basal and apical portion of each. *$p < 0.05$ and **$p < 0.025$; between control conjunctiva, basal and apical portion of pterygium. (B) Comparison of *KRT2*4 mRNA levels between three control conjunctiva tissues and 12 pterygium tissues at the basal and apical portion. *$p < 0.025$ between control conjunctiva, basal and apical portion of pterygium. Data are mean ± SD. Con: Control; PA: Apical portion of pterygium; PB: Basal portion of pterygium.

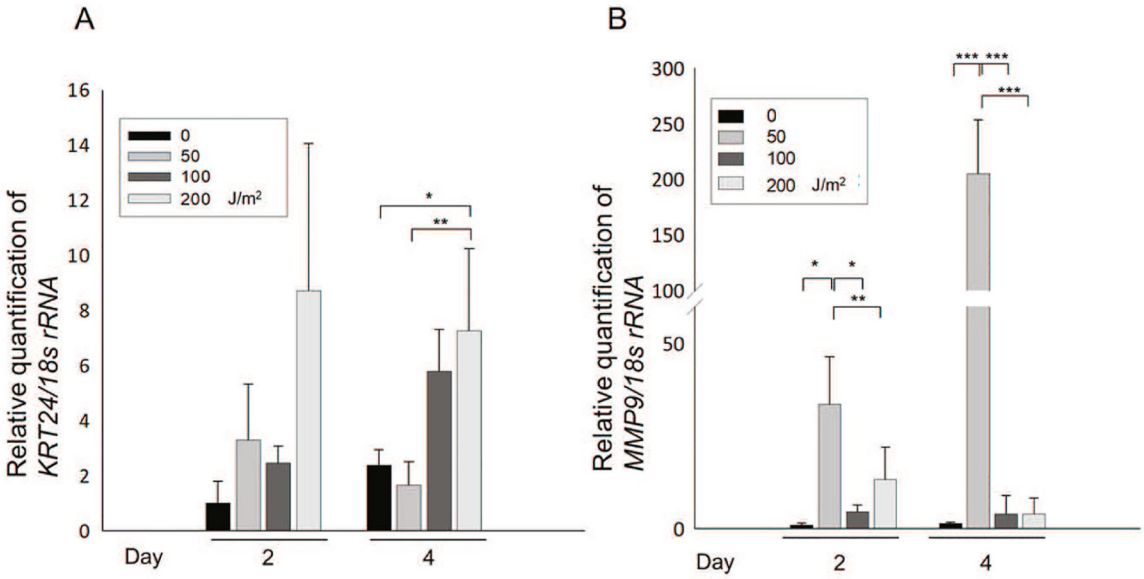

Fig. 4.

Expression of *MMP-9* (A) and *KRT24* (B) mRNA in HCEpC cells after UV-B exposure. As shown in Fig. 2A, after culturing for 2 and 4 days, the cells were collected and studied by a real-time RT-qPCR. (A) *KRT2*4 mRNA levels in HCEpCs exposed to 0–200 J/m2 UV-B. *p < 0.02, **p < 0.04 between 0 and 200 J/m^2 or 50 and 200 J/m^2 UV-B exposure on day 4. Data are mean ± SD (n = 6). (B) *MMP-*9 mRNA levels in HCEpCs exposed to 0–200 J/m^2 UV-B. *p < 0.007, **p < 0.05, ***p < 0.001 between 0 and 50 J/m^2, or 50 and 100 or 200 J/m^2 UV-B exposure on Day2 and 4. Data are mean ± SD (n = 6).

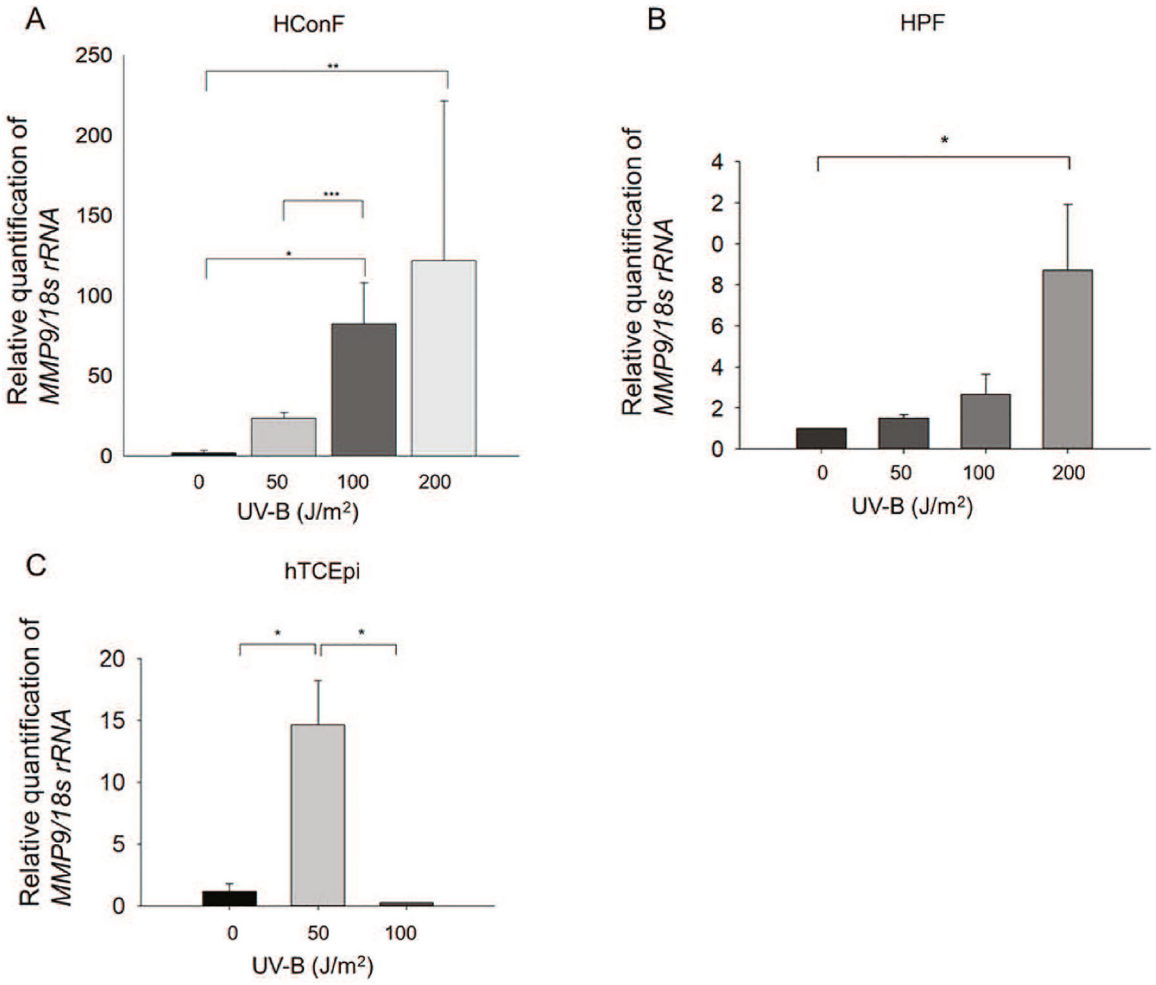

Fig. 5.

Expression of *MMP*-9 mRNA in HConFC, HPF and hTCEpi cells after UV-B exposure. (A) As shown in Fig. 2A, after culturing for 4 days, the cells were collected and studied by real-time RT-qPCR. *MMP*-9 mRNA levels in HConFCs exposed to 0–200 J/m^2 UV-B. *$p < 0.001$, **$p < 0.03$, ***$p < 0.005$ between 0 and 50 or 100 J/m^2 UV-B exposure. Data are mean ± SD (n = 6). (B) As shown in Fig. 2A, after culturing for 2 days, the cells were collected and studied by RT-qPCR. *MMP*-9 mRNA levels in HPFs exposed to 0–200 J/m^2 UV-B. *$p < 0.05$. Data are mean ± SD (n = 3). (C) As shown in Fig. 2B, after culturing for 2 days, the hTCEpis were collected and studied by RT-qPCR. *MMP*-9 mRNA levels in hTCEpis exposed to 0–100 J/m^2 UV-B. *$p < 0.025$. Data are mean ± SD (n = 3).

Table 1

Top 10 genes ranked by the greatest differences in log2 ratio between control and PA1–3.

PA1			PA2			PA3		
Probe Name	**Fold Chane Log2 ratio**	**Gene Symbol**	**Probe Name**	**Fold Change Log2 ratio**	**Gene Symbol**	**Probe Name**	**Fold Change Log2 ratio**	**Gene Symbol**
A 23 P4387	6.864	KRT24	A_23_P4387	4.877	KRT24	A_23_P40174	5.586	MMP9
A 33 P3311503	4.935	SMR3B	A_23_P93141	4.686	GSTA5	A_23_P7313	4.962	SPP1
A 23 P29965	4.632	SMR3B	A_33_P3273885	4.429		A_23_P18452	4.420	CXCL9
A 24 P870620	4.431	PTN	A_23_P40174	4.395	MMP9	A_24_P131589	4.162	CD86
A 23 P214144	4.250	COL10A1	A_32_P60065	4.255	F2RL2	A_33_P3329078	4.088	HBG1
A 23 P132760	3.977	TRH	A_23_P214300	4.146	GSTA2	A_21_P007321	4.007	
A 33 P3241269	3.837	CES1	A_23_P69030	3.689	COL8A1	A_32_P157927	3.880	
A 24 P626951	3.783		A_23_P7313	3.629	SPP1	A_21_P007446	3.816	
A 33 P3304668	3.780	COL1A1	A_33_P3364240	3.615	PAEP	A_19_P00809119	3.800	CASC15
A_33_P3281435	3.533		A_33_P233040	3.493	SERPINB11	A_24_P70183	3.453	MYH11

Table 2

Top 30 genes that showed significant changes of greater than 5.0-fold in common to 3 pterygium samples.

Gene Symbol	GenBank Accession	Gene Name	Ave(n = 3)	SD
KRT24	NM_019016	keratin 24	49.446	21.618
MMP9	NM_004994	matrix metallopeptidase 9 (gelatinase B, 92 kDa gelatinase, 92 kDa type IV collagenase)	24.214	10.393
PTN	NM_002825	pleiotrophin	12.844	7.892
GSTA5	NM_153699	glutathione S-transferase alpha 5	12.809	10.397
SMR3B	NM_006685	submaxillary gland androgen regulated protein 3B	12.722	15.471
CXCL9	NM_002416	chemokine (C-X-C motif) ligand 9	12.220	7.955
GSTA2	NM_000846	glutathione S-transferase alpha 2	11.350	5.891
COL8A1	NM_001850	collagen, type VIII, alpha 1	10.628	2.661
CES1	NM_001025195	carboxylesterase 1	10.570	3.885
COL10A1	NM_000493	collagen, type X, alpha 1	10.316	8.039
GZMA	NM_006144	granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3)	8.177	1.592
UCHL1	NM_004181	ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)	7.635	1.961
TMEM119	NM_181724	transmembrane protein 119	7.213	1.765
PTGFR	NM_001039585	prostaglandin F receptor (FP)	7.174	2.224
NFATC4	NM_001136022	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 4	7.068	1.801
PI16	NM_153370	peptidase inhibitor 16	6.765	1.703
COMP	NM_000095	cartilage oligomeric matrix protein	6.725	1.665
GDF10	NM_004962	growth differentiation factor 10	6.567	3.089
EPS15	AK129853	epidermal growth factor receptor pathway substrate 15	6.510	1.071
CES1	NM_001266	carboxylesterase 1	6.493	4.419
FAP	NM_004460	fibroblast activation protein, alpha	6.441	1.111
MYH11	NM_001040113	myosin, heavy chain 11, smooth muscle	6.330	1.886
SEMA3D	NM_152754	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3D	5.949	2.084
ITGBL1	NM_004791	integrin, beta-like 1 (with EGF-like repeat domains)	5.906	1.925
SERPINB11	NM_080475	serpin peptidase inhibitor, clade B (ovalbumin), member 11 (gene/pseudogene)	5.882	4.718
APOC1	NM_001645	apolipoprotein C-I	5.679	3.761
ARHGAP44	NM_014859	Rho GTPase activating protein 44	5.641	0.694
CYS1	NM_001037160	cystin 1	5.592	1.349
GJC1	NM_005497	gap junction protein, gamma 1, 45 kDa	5.567	1.120
HBA221:31	NM_000517	hemoglobin, alpha 2	5.496	3.325

Table 3

Gene ontology analysis that showed significant changes of greater than 5.0-fold in common to 3 pterygium samples.

GO ACCESSION	GO Term	p-value
GO:0031012	extracellular matrix	2.99E-33
GO:0044421	extracellular region part	9.94E-31
GO:0005576	extracellular region	2.85E-29
GO:0005578	proteinaceous extracellular matrix	2.35E-25
GO:0030198	extracellular matrix organization	7.38E-24
GO:0043062	extracellular structure organization	8.80E-24
GO:0022617	extracellular matrix disassembly	8.47E-17
GO:0005615	extracellular space	2.93E-16
GO:0001944	vasculature development	4.80E-16
GO:0001568	blood vessel development	5.50E-15
GO:0044420	extracellular matrix part	1.66E-14
GO:0030334	regulation of cell migration	9.65E-14
GO:0048731	system development	5.69E-13
GO:2000145	regulation of cell motility	8.80E-13
GO:0044259	multicellular organismal macromolecule metabolic process	9.34E-13
GO:0030574	collagen catabolic process	8.77E-13
GO:0051270	regulation of cellular component movement	1.51E-12
GO:0032963	collagen metabolic process	1.64E-12
GO:0072358	cardiovascular system development	1.87E-12
GO:0032501|GO:0050874	multicellular organismal process	1.69E-12

Table 4

Top 30 genes (a) and analysis of gene ontology features (b) that showed significant changes of smaller than 0.5-fold in common to 3 pterygium samples.

Gene Symbol	GenBank Accession	Gene Name	Ave(n = 3)	SD
DEFB4A	NM_004942	defensin, beta 4A	0.109	0.065
CEACAM5	NR_038428	chromosome X open reading frame 28	0.135	0.084
KRT32	NM_002278	keratin 32	0.159	0.082
RHCG	NM_016321	Rh family, C glycoprotein	0.163	0.060
NXPH4	NM_007224	neurexophilin 4	0.170	0.068
FOSB	NM_006732	FBJ murine osteosarcoma viral oncogene homolog B	0.173	0.080
RNF151	NM_174903	ring finger protein 151	0.189	0.083
TUBBP5	NR_027156	tubulin, beta pseudogene 5	0.195	0.204
C10orf99	NM_207373	chromosome 10 open reading frame 99	0.200	0.028
GDF15	NM_004864	growth differentiation factor 15	0.207	0.049
NWD1	NM_001007525	NACHT and WD repeat domain containing 1	0.214	0.114
CRNN	NM_016190	cornulin	0.215	0.205
SPINK2	NM_021114	serine peptidase inhibitor, Kazal type 2 (acrosin-trypsin inhibitor)	0.224	0.080
TRNP1	NM_001013642	TMF1-regulated nuclear protein 1	0.233	0.071
CCDC60	NM_178499	coiled-coil domain containing 60	0.234	0.006
MUC20	NM_001098516	mucin 20, cell surface associated	0.239	0.083
UCA1	NR_015379	urothelial cancer associated 1 (non-protein coding)	0.242	0.118
ATF3	NM_001040619	activating transcription factor 3	0.249	0.101
KRT34	NM_021013	keratin 34	0.251	0.113
ATF3	NM_001674	activating transcription factor 3	0.251	0.138
LOC100506810	NR_038856	uncharacterized LOC100506810	0.252	0.105
ZNF812	NM_001199814	zinc finger protein 812	0.255	0.053
FOS	NM_005252	FBJ murine osteosarcoma viral oncogene homolog	0.256	0.056
OAS1	NM_002534	2′–5′-oligoadenylate synthetase 1, 40/46 kDa	0.257	0.005
LINC00265	NR_026999	long intergenic non-protein coding RNA 265	0.261	0.014
CRYM	NM_001888	crystallin, mu	0.262	0.049
PADI1	NM_013358	peptidyl arginine deiminase, type I	0.262	0.129
ABO	NM_020469	ABO blood group (transferase A, alpha 1–3-N-acetylgalactosaminyltransferase; transferase B, alpha 1–3-galactosyltransferase)	0.265	0.049

Gene Symbol	GenBank Accession	Gene Name	Ave(n = 3)	SD
SNORA54	NR_002982	small nucleolar RNA, H/ACA box 54	0.267	0.087
TNFSF15	NM_005118	tumor necrosis factor (ligand) superfamily, member 15	0.268	0.096

Table 5

Gene ontology analysis that showed significant changes of smaller than 0.5-fold in common to 3 pterygium samples.

GO ACCESSION	GO Term	p-value
GO:0051607	defense response to virus	3.65E-06
GO:0071357	cellular response to type I interferon	3.94E-05
GO:0034340	response to type I interferon	4.53E-05
GO:0060337	type I interferon signaling pathway	3.41E-05
GO:0009615	response to virus	4.10E-04
GO:0016266	O-glycan processing	4.32E-04