

Expression of Inflammatory-Related NF κ B Genes in Iranian Patients with Pterygium: A Case-Control Study

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Pterygium is one of the most common eye conditions without any clear etiology. Some studies have suggested an association between sun exposure and pterygium, but others have proposed the role of genetic variations in its pathogenesis. To date, no study has investigated the association of inflammatory transcription factor, NF κ B genes with pterygium in the Middle East. We examined the changes in expression of 3 inflammatory related NF κ B1, NF κ B2, and RELA genes in patients with pterygium. Thirty patients with pterygium and 30 age and sex-matched controls were enrolled in this case-control study. None of the participants showed any clinical signs of inflammation in their conjunctiva. Demographic information was obtained and the expression levels of three genes including NF κ B1, NF κ B2, and RELA were measured in their conjunctiva by real-time RT-PCR using gene-specific primers. Mean expression level of NF κ B1, NF κ B2 and RELA genes in patients were 2.4 \pm 0.3, 1.9 \pm 0.5, and 1.8 \pm 0.4 times higher than normal subjects, respectively. Higher levels of gene expression were observed in individuals with more outdoor activity and sun exposure. Moreover, a significant correlation was observed between the expression levels of NF κ B2 and RELA genes, suggesting a possible NF κ B2- RELA heterodimer formation in patients with pterygium. This study has indicated a significant association between expressions of inflammatory-related NF κ B1, NF κ B2 and RELA genes, and pterygium. Further studies to verify the role of inflammation in the pathogenesis of pterygium, may provide new targets for managing pterygia.

Key words: Pterygium, inflammation, gene expression, NF-kappa B, real-time RT-PCR

Pterygium is a common ocular disorder characterized by the growth of a wing-like connective tissue on the cornea (1) that can cause irritation, foreign body sensation, and conjunctival

hyperemia. It also induces astigmatism and affects visual quality (1, 2). Pterygium occurrence is generally high in semi-dry regions, and its prevalence was reported to be 2% (3) while

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according to a cohort study in Iran the prevalence of pterygium in the urban population was 9.4% and 2.9% in one and both eyes, respectively (4).

Exposure to sunlight and genetic factors are the two main proposed contributors in the initiation and development of pterygium (5-7). Sunlight can induce inflammation, DNA damage, and secretion of growth factors, resulting in pterygium (8). So far, several reports have proposed the role of inflammation in pathogenesis, and post-surgery outcomes in pterygia (9-11). The nuclear factor kappa-light-chain-enhancer of activated B (*NF-κB*) family members including *NF-κB1* (*p50*), *NF-κB2* (*p52*), *RELA* (*p65*), *RELB* and *C-REL*, are the main mediators in initiating inflammation by inducing pro-inflammatory agents expression (12). Formation and activation of different dimers of *NF-κB* members mediate specific target gene expression in response to various stimuli (13). Various biological processes such as proliferation, apoptosis, differentiation, and migration are regulated through the *NF-κB* pathway with expression of more than 150 genes (14). Of the important relevant genes in pterygium are epithelial neutrophil activating peptide 78 (*CXCL5*), monocyte chemoattractant protein 1 (*CCL2*), and matrix metalloproteinases (*MMPs*) (15). A few studies suggested the role of *NF-κB* in cellular responses to UV damage and hyperosmotic stress (16, 17) that might have a role in pterygium.

Siak et al., for the first time reported *NF-κB* pathway activation in pterygium in Singaporean patients (18). Another study at 2017 showed elevated levels of local pro-inflammatory cytokine and nitric oxide responses in pterygium of Algerian patients (19). Recently, a few studies have concluded that despite the role of *NF-κB* family members as an initiator of inflammation, their inhibition after the initial inflammatory insult, may prolong the process of inflammation and delay tissue repair (20, 21).

In this study, we evaluated the probable

associations between the mean expression levels of *NFκB1*, *NFκB2* and *RELA* genes in the pterygium. Amongst all family members, these three proteins are more prevalent in activated form of NFκB heterodimers (12, 22). Dysregulation or aberrant expression of NFκB proteins were shown to be linked with neoplasms, autoimmune and inflammatory disorders (23-26). Therefore, the aim of this study was to examine the expression of these inflammatory-related genes in patients with pterygia in a case-control study.

Material and methods

Study subjects

Specimens from 30 patients with pterygium and 30 controls were collected from Khalili Hospital, Shiraz, Iran between 2016 and 2017. Inclusion criteria were no history of prior ocular surgery and taking topical ocular medications other than lubricants. All patients with history of using anti-glaucoma medications, inflamed pterygium or pseudopterygium were excluded. Inflamed conjunctiva was considered as hyperemia, membrane, pseudomembrane, and, follicular or papillary reaction. A true pterygium was considered if it had edges that could be elevated with forceps or under which a probe could be passed and usually arose from a pinguecula. The control subject's specimen consisted of 2×2 mm supranasal conjunctiva, taken from the patients who had undergone cataract extraction, at the end of surgery. The participants were examined before surgery and the controls had no ocular surface disease, pterygium or pinguecula. They were asked about their occupation and amount of sun exposure. Written informed consent was obtained from all participants. The study was approved by the local school of medicine research ethics committee, Shiraz University of Medical Sciences.

RNA isolation and real time PCR quantification of *NFκB1*, *NFκB2* and *RELA* expression

RNeasy Plus Mini Kit (QIAGEN, Netherlands) was used to isolate total RNA from case and control groups. From each sample, 1 µg of total RNA was converted into cDNA (final volume 20 µl) using QuantiTect reverse transcription kit (QIAGEN, Netherlands). Quantitative real time PCR of the synthesized cDNA molecules, was performed using ABI5700 sequence detection system (Applied Biosystems, USA). *GAPDH* was used as the internal control gene. All experimental steps were performed based on the manufacturer is

instructions. For real time PCR amplification, 1 µl of cDNA, 10 pmol of each specific primer (1 µl of each primer), 10 µl of 2× SYBR Green master mix, were mixed in a 20 µl final reaction volume. For each sample, the amplification was performed three times. We calculate the mean ± SD for each group, the individual data points, using $2^{-\Delta CT}$ as previously described (27). Primers and their sequences are shown in table 1.

Statistical analysis

Table 1. Primer sequences for studied genes

Gene	Primer sequences (5' → 3')	Annealing temperature (°C)	Amplicon size (bp)
<i>NFκB1</i>	F: CTATGACCTGGATGACTCTT R: ATGTCTCCTTGTGCTAGTAA	60	165
<i>NFκB2</i>	F: CCAGTGATGGCTCCTT R: AACCTCAATGTCATCTTTCTG	60	182
<i>RELA</i>	F: CTGCCAGATACAGACGAT R: GGGTCCGCTGAAAGG	60	99
<i>GAPDH</i>	F: CCTAGATTATTCTCTGATTTGGT R: ATGTAGTTGAGGTCAATGAAG	60	115

Table 2. Demographic information of studied subjects

Parameters	Age (Years ±SD)	Males N(%)	Females N(%)	Outdoor occupation N(%)	Sun exposure (hr/day)
Cases	53 ± 3.8	15 (50%)	15 (50%)	25 (83%)	11 ± 2
Controls	55 ± 1.9	18 (60%)	12 (40%)	15 (50%)	6.5 ± 1.5

Table 3. Mean expression of NFκB1, NFκB2 and RELA

Gene	Mean ± SD (Case)	Mean ± SD (Control)	P value
<i>NFκB1</i>	0.22 ± 0.13	0.10 ± 0.06	P<0.0001
<i>NFκB2</i>	0.18 ± 0.15	0.08 ± 0.06	P<0.0001
<i>RELA</i>	0.25 ± 0.19	0.13 ± 0.10	P<0.0001

Qualitative data were described in number (percent) and quantitative variables were expressed as the mean values ± SD. Normality of the data was checked by Kolmogorov-Smirnov test. Data of mean gene expression were analyzed by student t-test. Mean sun exposure was analyzed by student t-

test. The correlations between expression levels of genes were assessed by Pearson’s correlation test. P value <0.01 was considered to be statistically significant.

Results

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The pterygium patients were 15 males and 15 females with mean age of 53, and the control group consisted of 18 males and 12 females with mean age of 55 years. Table 2 shows the demographic information of the subjects. Outdoor occupations included agriculture, construction, gardening, and fishing. The amount of sun exposure was stated by the participants. Study groups were matched for age and gender. Outdoor occupation rate was analyzed by chi-square test, which was statistically significant between the two groups ($P= 0.003$). Mean sun exposure between the two groups was evaluated by student t-test and was statistically significant ($P= 0.002$).

The relative genes' expressions in patients versus controls are shown in figure 1. Quantification of *NFκB1*, *NFκB2* and *RELA* expression showed a significant difference between cases and controls ($P< 0.0001$) (Table 3).

Moreover, a significant correlation was found between *NFκB2* and *RELA* expressions in patients as verified by Pearson's correlation test (2-tailed). No statistically significant correlation was found between expressions of *NFκB2* and *NFκB1* or *RELA* and *NFκB1*.

Discussion

A few studies have suggested that pterygium should be classified as a degenerative process (28), while others consider it as an unusual growth disorder (29, 30), or a dysfunction in limbal stem cells (31).

The results of epidemiologic experiments revealed some associations between pterygium with other sun-related eye disorders, such as cataract, basal cell carcinoma, and spheroidal degeneration (31-34). In vitro experiments showed the major role of ultra violet ray in initiating pterygium (35-37). Higher levels of vitamin D and higher occurrence of pterygium in men with more outdoor activities can be a confirmation for the indisputable role of sun exposure in the pathogenesis of pterygium (38); which are consistent with the results of our study.

Some reports proposed genetic susceptibility to pterygium. For instance, polymorphisms in genes of DNA repair system such as *Ku70* in non-homologous end-joining repair system (39) and human 8-oxoguanine glycosylase I (*hOGG1*) (40) can be considered as a genetic factor of predisposition to pterygium. Sometimes, polymorphisms in DNA repair system elements can predisposition to pterygium. Sometimes, polymorphisms in DNA repair system elements can be positive. For example, polymorphism of

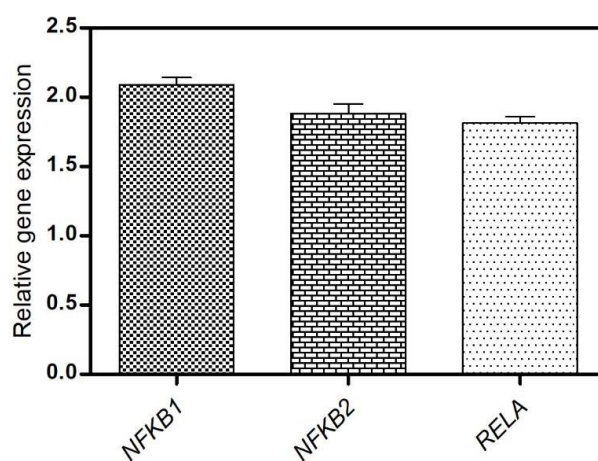


Fig. 1. Relative genes expression in patients versus controls.

X-ray repair cross complementary 1 (*XRCC1*) gene might lead to decreased chance of pterygium development (41). In a study, increased levels of transforming growth factor (*TGF-β1*) expression in pterygium tissue of different atopic cases suggested the associations between this growth factor and pathogenesis of pterygium (42).

In this study, we attempted to find the role of inflammation in this disease. Hence, we evaluated the expression levels of three main genes of *NFκB* family including *NFκB1*, *NFκB2* and *RELA* in patients with pterygium and controls. Selecting *NFκB* family was due to their central role as an initiator of different inflammation pathways (12). Our results showed significant differences in expression levels of three *NFκB* family genes among the participants. Mean expression levels of *NFκB1*, *NFκB2* and *RELA* genes were 2.4 ± 0.3 , 1.9 ± 0.5 , and 1.8 ± 0.4 times higher in patients with pterygium in comparison with controls, respectively. These findings are consistent with the finding reported by Siak et al (18). A study by Torres et al., 2011, on 21 cases and 13 controls revealed that patients with pterygium had alterations in *NFκB* pathways (43), which is in line with our findings. They also showed higher levels of *NFκB* expression in ipsilateral pterygium-free conjunctiva. Investigating the other eye of the unilateral pterygium patients and following them to see any pterygium development can clarify the causative role of *NFκB* expression in pterygium that can be targeted pharmacologically; by retinoic acid, for example.

As noted by Siak et al., (18), Torres et al., (43) and also in this study, participants with clinical inflammation were excluded from the study. This signifies the presence of subclinical inflammation.

Further analysis of demographic information of patients indicated a significant correlation between outdoor activity and *NFκB* family expression levels. Most of the individuals with pterygium had more outdoor activities and sun

exposure in comparison with the controls, suggesting the correlation between sun exposure, inflammation and pathogenesis of pterygium.

However, more studies are needed to verify the role of inflammation in the etiology of pterygium.

We also found a positive correlation between *NFκB2* and *RELA* genes in the pterygium patients. These two proteins can form NFκB heterodimer. Activated forms of NFκB can either contain NFκB1 and *RELA* or *NFκB2* and *RELA* proteins. A heterodimer including *NFκB2* and *RELA* contains the necessary transactivation domains that can lead to gene induction and initiation of inflammation cascades. Further investigations are required to confirm the NFκB heterodimer formation and its clinical importance in the etiology of pterygium.

In conclusion, in this study, by measuring *NFκB* family expression levels, as the key players in inflammatory conditions, we provided evidence for a possible association between inflammation and pterygium and found generalizability of this expression in Iranian population. We also showed a positive correlation between sun exposure and expression of inflammatory elements. This study showed a correlation between inflammation through *NFκB* family members and pterygium that indicates a subclinical inflammation in pterygium that might be a target for therapy. In other words, even in uninflamed pterygia, anti-inflammatory drugs might stop the progression of the disease. Further investigations including measuring NFκB protein levels and other markers of inflammations are recommended.

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Conflict of interest

The authors declare no conflict of interest.

References

- Bianchi E, Scarinci F, Grande C, et al. Immunohistochemical Profile and VEGF, TGF- β and PGE2 in Human Pterygium and Normal Conjunctiva: Experimental Study and Review of the Literature. *Int J Immunopathol Pharmacol* 2012;25:607-15.
- Hasegawa A, Hanaoka M, Murakoshi T. Continuous fetal head flexion as a marker for prenatal diagnosis of lethal multiple pterygium syndrome: a case report. *J Med Ultrason*(2001) 2017;44:271-3.
- Detorakis ET, Spandidos DA. Pathogenetic mechanisms and treatment options for ophthalmic pterygium: trends and perspectives (Review). *Int J Mol Med* 2009;23:439-47.
- Rezvan F, Hashemi H, Emamian MH, et al. The prevalence and determinants of pterygium and pinguecula in an urban population in Shahroud, Iran. *Acta Med Iran* 2012;50:689-96.
- Yam JC, Kwok AK. Ultraviolet light and ocular diseases. *Int Ophthalmol* 2014;34:383-400.
- Romano V, Steger B, Kovacova A, et al. Further evidence for heredity of pterygium. *Ophthalmic Genet* 2016;37:434-6.
- Anguria P, Kitinya J, Ntuli S, et al. The role of heredity in pterygium development. *Int J Ophthalmol* 2014;7:563-73.
- Sul S, Korkmaz S, Novruzlu S. Seasonal effects on pterygium surgery outcome: implications for the role of sunlight exposure. *Cornea* 2014;33:504-6.
- Kheirkhah A, Casas V, Sheha H, et al. Role of conjunctival inflammation in surgical outcome after amniotic membrane transplantation with or without fibrin glue for pterygium. *Cornea* 2008;27:56-63.
- Solomon A, Li D-Q, Lee S-B, et al. Regulation of collagenase, stromelysin, and urokinase-type plasminogen activator in primary pterygium body fibroblasts by inflammatory cytokines. *Invest Ophthalmol Vis Sci* 2000;41:2154-63.
- Ebrahimi ME, Kordi-Tamandani DM, Arish M. A novel approach to investigation of the pathogenesis of pterygium based on assessment of promoter hyper-methylation and expression profile of CTLA4 gene: A credible report of CTLA4 gene expression in human eye tissue. *Gene* 2016;583:130-3.
- Tak PP, Firestein GS. NF-kappaB: a key role in inflammatory diseases. *J Clin Invest* 2001;107:7-11.
- Ghosh S, May MJ, Kopp EB. NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. *Annu Rev Immunol* 1998;16:225-60.
- Pahl HL. Activators and target genes of Rel/NF-kappaB transcription factors. *Oncogene* 1999;18:6853-66.
- Basak S, Hoffmann A. Crosstalk via the NF-kappaB signaling system. *Cytokine Growth Factor Rev* 2008;19:187-97.
- Nemeth ZH, Deitch EA, Szabo C, et al. Hyperosmotic stress induces nuclear factor-kappaB activation and interleukin-8 production in human intestinal epithelial cells. *Am J Pathol* 2002;161:987-96.
- Matsuo H, Tamura M, Kabashima N, et al. Prednisolone inhibits hyperosmolarity-induced expression of MCP-1 via NF-kappaB in peritoneal mesothelial cells. *Kidney Int* 2006;69:736-46.
- Siak JJ, Ng SL, Seet LF, et al. The nuclear-factor kappaB pathway is activated in pterygium. *Invest Ophthalmol Vis Sci* 2011;52:230-6.
- Zidi S, Bediar-Boulaneb F, Belguendouz H, et al. Local pro-inflammatory cytokine and nitric oxide responses are elevated in patients with pterygium. *Int J Immunopathol Pharmacol* 2017;30:395-405.
- Ghosh S, Hayden MS. New regulators of NF-kappaB in inflammation. *Nat Rev Immunol* 2008;8:837-48.
- Lawrence T, Gilroy DW, Colville-Nash PR, et al. Possible new role for NF-kappaB in the resolution of inflammation. *Nat Med* 2001;7:1291-7.
- Esteban V, Ruperez M, Vita JR, et al. Effect of simultaneous blockade of AT1 and AT2 receptors on the NFkappaB pathway and renal inflammatory response. *Kidney Int Suppl* 2003:S33-8.
- Meffert MK, Chang JM, Wiltgen BJ, et al. NF-kappa B functions in synaptic signaling and behavior. *Nat Neurosci* 2003;6:1072-8.
- Azizan N, Suter MA, Liu Y, et al. RAGE maintains high levels of NFkappaB and oncogenic Kras activity in pancreatic cancer. *Biochem Biophys Res Commun* 2017;493:592-7.
- Garg B, Giri B, Modi S, et al. NFkappaB In Pancreatic Cancer

Stroma Suppresses Anti-Tumor Immunity. *Pancreatology* 2017;17.

26. Camp SM, Ceco E, Evenoski CL, et al. Unique Toll-Like Receptor 4 Activation by NAMPT/PBEF Induces NFκB Signaling and Inflammatory Lung Injury. *Sci Rep* 2015;5:13135.

27. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* 2008;3:1101-8.

28. Tradjutrismo N. Pterygium: degeneration, exuberant wound healing or benign neoplasm? . *Universa Medicina* 2016;28:179-87.

29. Tan DT, Lim AS, Goh HS, et al. Abnormal expression of the p53 tumor suppressor gene in the conjunctiva of patients with pterygium. *Am J Ophthalmol* 1997;123:404-5.

30. Di Girolamo N, Chui J, Coroneo MT, et al. Pathogenesis of pterygia: role of cytokines, growth factors, and matrix metalloproteinases. *Prog Retin Eye Res* 2004;23:195-228.

31. Chui J, Coroneo MT, Tat LT, et al. Ophthalmic pterygium: a stem cell disorder with premalignant features. *Am J Pathol* 2011;178:817-27.

32. Moran DJ, Hollows FC. Pterygium and ultraviolet radiation: a positive correlation. *Br J Ophthalmol* 1984;68:343-6.

33. Threlfall TJ, English DR. Sun exposure and pterygium of the eye: a dose-response curve. *Am J Ophthalmol* 1999;128:280-7.

34. McCarty CA, Fu CL, Taylor HR. Epidemiology of pterygium in Victoria, Australia. *Br J Ophthalmol* 2000;84:289-92.

35. Di Girolamo N, Kumar RK, Coroneo MT, et al. UVB-mediated induction of interleukin-6 and -8 in pterygia and cultured human pterygium epithelial cells. *Invest Ophthalmol*

Vis Sci 2002;43:3430-7.

36. Di Girolamo N, Coroneo MT, Wakefield D. UVB-elicited induction of MMP-1 expression in human ocular surface epithelial cells is mediated through the ERK1/2 MAPK-dependent pathway. *Invest Ophthalmol Vis Sci* 2003;44:4705-14.

37. Di Girolamo N, Coroneo M, Wakefield D. Epidermal growth factor receptor signaling is partially responsible for the increased matrix metalloproteinase-1 expression in ocular epithelial cells after UVB radiation. *Am J Pathol* 2005;167:489-503.

38. Kara N, Ceri S. Vitamin D level in patients with pterygium. *Arq Bras Oftalmol* 2017;80:229-33.

39. Tsai YY, Bau DT, Chiang CC, et al. Pterygium and genetic polymorphism of DNA double strand break repair gene Ku70. *Mol Vis* 2007;13:1436-40.

40. Kau HC, Tsai CC, Hsu WM, et al. Genetic polymorphism of hOGG1 and risk of pterygium in Chinese. *Eye (Lond)* 2004;18:635-9.

41. Chiang CC, Tsai YY, Bau DT, et al. Pterygium and genetic polymorphisms of the DNA repair enzymes XRCC1, XPA, and XPD. *Mol Vis* 2010;16:698-704.

42. Shayegan MR, Khakzad MR, Gharaee H, et al. Evaluation of transforming growth factor-beta1 gene expression in pterygium tissue of atopic patients. *J Chin Med Assoc* 2016;79:565-9.

43. Torres J, Enriquez-de-Salamanca A, Fernandez I, et al. Activation of MAPK signaling pathway and NF-κB activation in pterygium and ipsilateral pterygium-free conjunctival specimens. *Invest Ophthalmol Vis Sci* 2011;52:5842-52.