# Expression of Inflammatory-Related NFκB Genes in Iranian Patients with Pterygium: A Case-Control Study

Seyed Mohammad Salar Zaheryani<sup>1</sup>, Mohammad Essmail Ebrahimi<sup>1</sup>, Abdollah Kasaei<sup>2</sup>, Amir Roointan<sup>3</sup>, Mahmood Nejabat<sup>1\*,</sup> Mehdi Dianatpour<sup>2</sup>, Meisam Ghanbari<sup>1</sup>, Mohammad Reza Talebnejad<sup>1</sup>, Fakhraddin Naghibalhossaini<sup>4</sup>

1. Poostchi Ophthalmology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran.

2. Stem Cells Technology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran.

3. Department of Medical Biotechnology, School of Advanced Medical Sciences and Technologies, Shiraz

University of Medical Sciences, Shiraz, Iran.

4. Biochemistry Department, Shiraz University of Medical Sciences, Shiraz, Iran.

#### Submmited 15 June 2018; Accepted 18 October 2018; Published 22 October 2018

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* $\kappa$ *B* genes with pterygium in the Middle East. We examined the changes in expression of 3 inflammatory related *NF* $\kappa$ *B1*, *NF* $\kappa$ *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* $\kappa$ *B1*, *NF* $\kappa$ *B2*, and *RELA* were measured in their conjunctiva by real-time RT-PCR using gene-specific primers. Mean expression level of *NF* $\kappa$ *B1*, *NF* $\kappa$ *B2* and *RELA* genes in patients were 2.4±0.3, 1.9± 0.5, and 1.8±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* $\kappa$ *B1*, *NF* $\kappa$ *B2* and *RELA* genes, suggesting a possible NF $\kappa$ B2- RELA heterodimer formation in patients with pterygium. This study has indicated a significant association between expressions of inflammatory-related *NF* $\kappa$ *B1*, *NF* $\kappa$ *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

\*Corresponding author: Poostchi Ophthalmology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran. E Mail: nejabatm@sums.ac.ir

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- $\kappa B$ ) family members including NF- $\kappa B1$  (p50), NF- $\kappa B2$  (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 NFκВ members mediate specific target gene expression in response to various stimuli (13). Various biological processes such as proliferation, differentiation, and apoptosis, migration are regulated through the  $NF-\kappa B$  pathway with expression of more than 150 genes (14). Of the important relevant genes in pterygium are epithelial neutrophil activating peptide 78 (CXCL5), monocyte chemotactic protein 1 (CCL2), and matrix metalloproteinases (MMPs) (15). A few studies suggested the role of  $NF - \kappa B$  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-\kappa B* 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-\kappa B* 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  $NF\kappa B1$ ,  $NF\kappa B2$  and *RELA* genes in the pterygium. Amongst all family members, these three proteins are more prevalent in activated form of NF $\kappa$ B heterodimers (12, 22). Dysregulation or aberrant expression of NF $\kappa$ B 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, psudomembrane, 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 the controls had no ocular surface and 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

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#### Pterygium and expression of NFkB-related genes

RNeasy Plus Mini Kit (QIAGEN, Netherlands) was used to isolate total RNA from case and control groups. From each sample, 1  $\mu$ g of total RNA was converted into cDNA (final volume 20  $\mu$ l) using QuantiTect reverse transcription kit (QIAGEN, Netherlands). Quantitative real time PCR of the synthesized cDNA molecules, was performed using AB15700 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  $\pm$  SD for each group,the individual data points, using 2<sup>- $\Delta$ CT</sup> 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 $(5' \rightarrow 3')$	Annealing	Amplicon			
		temperature	size			
		(°C)	(bp)			
ΝΓκΒΙ	F: CTATGACCTGGATGACTCTT	60	165			
	R: ATGTCTCCTTGTGCTAGTAA					
ΝΓκΒ2	F: CCAGTGATGGCTCCTT	60	182			
	R: AACCTCAATGTCATCTTTCTG					
RELA	F: CTGCCAGATACAGACGAT	60	99			
	R: GGGTCCGCTGAAAGG					
	F: CCTAGATTATTCTCTGATTTGGT					
GAPDH	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	$53 \pm 3.8$	15 (50%)	15 (50%)	25 (83%)	$11 \pm 2$			
Controls	$55\pm1.9$	18 (60%)	12 (40%)	15 (50%)	$6.5 \pm 1.5$			

<b>Table 3.</b> Mean expression of NF $\kappa$ B1, NF $\kappa$ B2 and RELA							
Gene	Mean ± SD (Case)	Mean ± SD (Control)	P value				
ΝϜκΒ1	$0.22 \pm 0.13$	$0.10\pm0.06$	P<0.0001				
ΝϜκΒ2	$0.18\pm0.15$	$0.08\pm0.06$	P<0.0001				
RELA	$0.25\pm0.19$	$0.13\pm0.10$	P<0.0001				

Qualitative data were described in number (percent) and quantitative variables were expressed as the mean values  $\pm$  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

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 *NF* $\kappa$ *B1*, *NF* $\kappa$ *B2* and *RELA* expression showed a significant difference between cases and controls (P< 0.0001) (Table 3).

Moreover, a significant correlation was found between  $NF\kappa B2$  and *RELA* expressions in patients as verified by Pearson's correlation test (2-tailed). No statistically significant correlation was found between expressions of  $NF\kappa B2$  and  $NF\kappa B1$  or *RELA* and  $NF\kappa B1$ .

# 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 nonhomologous 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



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-\beta1*) 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  $NF\kappa B$ family including  $NF\kappa B1$ ,  $NF\kappa B2$  and RELA in patients with pterygium and controls. Selecting  $NF\kappa B$  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  $NF\kappa B$  family genes among the participants. Mean expression levels of  $NF\kappa B1$ ,  $NF\kappa B2$  and RELA genes were 2.4±0.3, 1.9± 0.5, and 1.8±0.4 times higher in patients with pterygium comparison with in 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  $NF\kappa B$  pathways (43), which is in line with our findings. They also showed higher levels of  $NF\kappa B$  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  $NF\kappa B$  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  $NF\kappa B$  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  $NF\kappa B2$  and *RELA* genes in the pterygium patients. These two proteins can form NF $\kappa$ B heterodimer. Activated forms of NF $\kappa$ B can either contain NF $\kappa$ B1 and RELA or NF $\kappa$ B2 and RELA proteins. A heterodimer including NF $\kappa$ B2 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 NF $\kappa$ B heterodimer formation and its clinical importance in the etiology of pterygium.

In conclusion, in this study, by measuring  $NF\kappa B$  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  $NF\kappa B$  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 NFkB protein levels and other markers of inflammations are recommended.

#### Acknowledgment

This study was supported by a grant from the Deputy Dean of Shiraz School of Medicine (Grant # 11591) and sponsored by Vice Chancellor for research, Shiraz University of Medical Sciences. The authors thank the following: Dr Nahid Hemmatian Boroujeni for her productive comments, the staff of operating room of Khalili Hospital, Shiraz, Iran to Dr Iman Jamhiri. For his utmost cooperation, and to Mr. H. Argasi of the Research Consultation Center (RCC) of Shiraz University of Medical Sciences for his invaluable assistance in editing this manuscript.

#### **Conflict of interest**

The authors declare no conflict of interest.

#### References

1. Bianchi E, Scarinci F, Grande C, et al. Immunohistochemical Profile and VEGF, TGF- $\beta$  and PGE2 in Human Pterygium and Normal Conjunctiva: Experimental Study and Review of the Literature. Int J Immunopathol Pharmacol 2012;25:607-15.

2. 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.

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.

5. Yam JC, Kwok AK. Ultraviolet light and ocular diseases. Int Ophthalmol 2014;34:383-400.

6. Romano V, Steger B, Kovacova A, et al. Further evidence for heredity of pterygium. Ophthalmic Genet 2016;37:434-6.

7. 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.

9. 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.

10. 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.

11. 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.

12. Tak PP, Firestein GS. NF-kappaB: a key role in inflammatory diseases. J Clin Invest 2001;107:7-11.

13. 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.

14. Pahl HL. Activators and target genes of Rel/NF-kappaB transcription factors. Oncogene 1999;18:6853-66.

15. Basak S, Hoffmann A. Crosstalk via the NF-kappaB signaling system. Cytokine Growth Factor Rev 2008;19:187-97.

16. 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.

17. Matsuo H, Tamura M, Kabashima N, et al. Prednisolone inhibits hyperosmolarity-induced expression of MCP-1 via NFkappaB in peritoneal mesothelial cells. Kidney Int 2006;69:736-46.

18. 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.

19. Zidi S, Bediar-Boulaneb F, Belguendouz H, et al. Local proinflammatory cytokine and nitric oxide responses are elevated in patients with pterygium. Int J Immunopathol Pharmacol 2017;30:395-405.

20. Ghosh S, Hayden MS. New regulators of NF-kappaB in inflammation. Nat Rev Immunol 2008;8:837-48.

21. 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.

22. 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.

23. Meffert MK, Chang JM, Wiltgen BJ, et al. NF-kappa B functions in synaptic signaling and behavior. Nat Neurosci 2003;6:1072-8.

24. 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.

25. Garg B, Giri B, Modi S, et al. NFkB In Pancreatic Cancer

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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 NFkappaB 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.

 Tradjutrisno 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.

 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. UVBmediated 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 MAPKdependent 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.

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-kappaB activation in pterygium and ipsilateral pterygium-free conjunctival specimens. Invest Ophthalmol Vis Sci 2011;52:5842-52.