JBUON 2018; 23(3): 826-831
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
E-mail: editorial_office@jbuon.com

ORIGINAL ARTICLE

Deregulation of p53/survivin apoptotic markers correlated to PTEN expression in pterygium neoplastic cells

Kallirroi Konstantopoulou[1]*, Evangelos Tsiambas[2,3]*, Evangelia Baliou[3], Andreas C. Lazaris[3], Nikolaos Kavantzas[3], Andreas Karameris[4], Panagiotis P. Fotiades[3], Ioannis Asproudis[5], Vasileios Ragos[6], George Vilaras[3], Efstratios Patsouris[3], Kallirroi Konstantopoulou[1]*, Evangelos Tsiambas[2,3]*, Evangelia Baliou[3], Andreas C. Lazaris[3], Nikolaos Kavantzas[3], Andreas Karameris[4], Panagiotis P. Fotiades[3], Ioannis Asproudis[5], Vasileios Ragos[6], George Vilaras[3], Efstratios Patsouris[3]

[1]Department of Ophthalmology, General Hospital, Kalamata, Greece; [2]Department of Immunohistochemistry-Molecular Biology, 401 GAH, Athens, Greece; [3]Department of Pathology, Medical School, University of Athens, Athens, Greece; [4]Department of Pathology, 417 VA Hospital, Athens, Athens, Greece; [5]Department of Ophthalmology, Medical School, University of Ioannina, Ioannina, Greece; [6]Department of Maxillofacial Surgery, Medical School, University of Ioannina, Ioannina, Greece

*These authors contributed equally to this study

Summary

Purpose: *Pterygium is a distinct clinicopathological entity characterized by degenerated and neoplastic-like features. Concerning its rise on normal conjunctiva epithelia, the role of specific gene deregulations including apoptotic/anti-apoptotic factors and significant suppressor genes in signaling transduction pathways is under investigation. In the current study, we co-analyzed p53, survivin and PTEN proteins in pterygia and normal conjunctiva.*

Methods: *Using a liquid-based cytology assay, 50 cell specimens were obtained by a smooth scraping on conjunctiva epithelia and fixed accordingly. Among them, 38 were pterygia and the remaining (n=12) normal epithelia (control group). Immunocytochemistry assays were implemented on the corresponding slides by applying ani-p53, survivin, and PTEN antibodies. Digital image analysis was performed for evaluating objectively the corresponding immunostaining intensity levels.*

Results: *The majority of the examined pterygia cases overexpressed the markers p53:22/38-57.9%, survivin:30/38-78.9%, and PTEN:25/38-65.7%. Interestingly, overall p53/PTEN co-expression was found to be statistically significant (p=0.022).*

Conclusions: *Survivin overexpression leads to an increased anti-apoptotic activity playing a central molecular role in the pathogenesis and progression of pterygia. Furthermore, although p53 expression is observed in these lesions, its impact seems to be low compared to survivin's influence on them. Additionally, the role of PTEN in the process is potentially significant providing a suppressor balance to the p53/survivin complex.*

Key words: *apoptosis, cytology, genes, image analysis, pterygia*

Introduction

Pterygium represents a frequent and partially unexplored epithelial lesion affecting the conjunctiva [1]. High levels of ultraviolet (UV) radiation exposure seem to be the main pathogenetic factor. Increased oxidative DNA damage due to aberrant UV influence acts as a central mechanism leading

Correspondence to: Evangelos Tsiambas, MD, MSc, PhD. 17 Patriarchou Grigoriou E΄ Street, Ag. Paraskevi, 153 41 Athens, Greece. Fax: +30 210 6526259, E-mail: tsiambasecyto@yahoo.gr
Received: 15/11/2017; Accepted: 08/12/2017

to an epithelial micro-enviroment transformation [2]. Concerning the morphological changes that modify normal conjunctiva epithelia, pterygia demonstrate an abnormal, mainly triangular shape growth pattern, affecting the cornea. Although the lesion is generally considered to be benign, a grade of progressive dysplasia combined with inflammation and sometimes a tissue invasive pattern has been reported. In fact, categorization of pterygium as lesion is difficult because of its nature and progression as a disease. Degeneration of the normal conjunctiva and localized neoplastic entity should be two different but closely related aspects of the same lesion [3].

Molecular analyses regarding pterygia have shown that deregulation of critical genes - implicated in cell proliferation, apoptosis and also signal transduction to the nucleus - are potentially involved in the progression of the lesion. Among them, survivin and p53 are main regulators of cell and tissue homeostasis due to their influence in the apoptotic pathways [4]. Survivin (gene locus: 17q25) is a member of the inhibitor of apoptosis (IAP) gene family which counteracts apoptosis by inhibiting the activity of initiators and effectors caspases. Survivin associates with the microtubules in the mitotic spindle and antagonizes mitochondrial-dependent apoptosis [5]. Survivin is also involved in cell division. In fact, survivin demonstrates a high expression at G2/M phase and declines rapidly in G1 phase of the cell cycle. On the other hand, p53 - a nuclear phosphoprotein - is a central genome gate-keeper (gene locus:17p13). It acts as a transcription factor for crucial genes, inhibiting abnormal DNA replication and regulating apoptosis. Mutant p53 due to somatic gene mutations leads to a nuclear-based protein overexpression pattern transforming its role from a suppressor to an oncogene [6]. In conjunction to the previous referred genes, *PTEN* (gene locus:10q23.3-phosphatase and tensin homolog deleted in chromosome 10) is a tumor suppressor gene that is deleted, mutated or epigenetically hypermethylated in a variety of human malignancies. PTEN acts as a negative regulator of this specific pathway [7]. Normal expression of PTEN induces growth suppression by promoting cell cycle arrest. It is also correlated with decreased levels and nuclear localization of cyclin D1 regulated by AKT that positively induces cell cycle [8]. In the current research study, we intended to explore the impact of the combined p53, PTEN, and survivin protein expression in pterygia epithelia.

Methods

Cell substrates

For the purposes of our study, 50 cell samples were obtained by applying a smooth scraping on the corresponding conjunctiva epithelia. The mean patient age

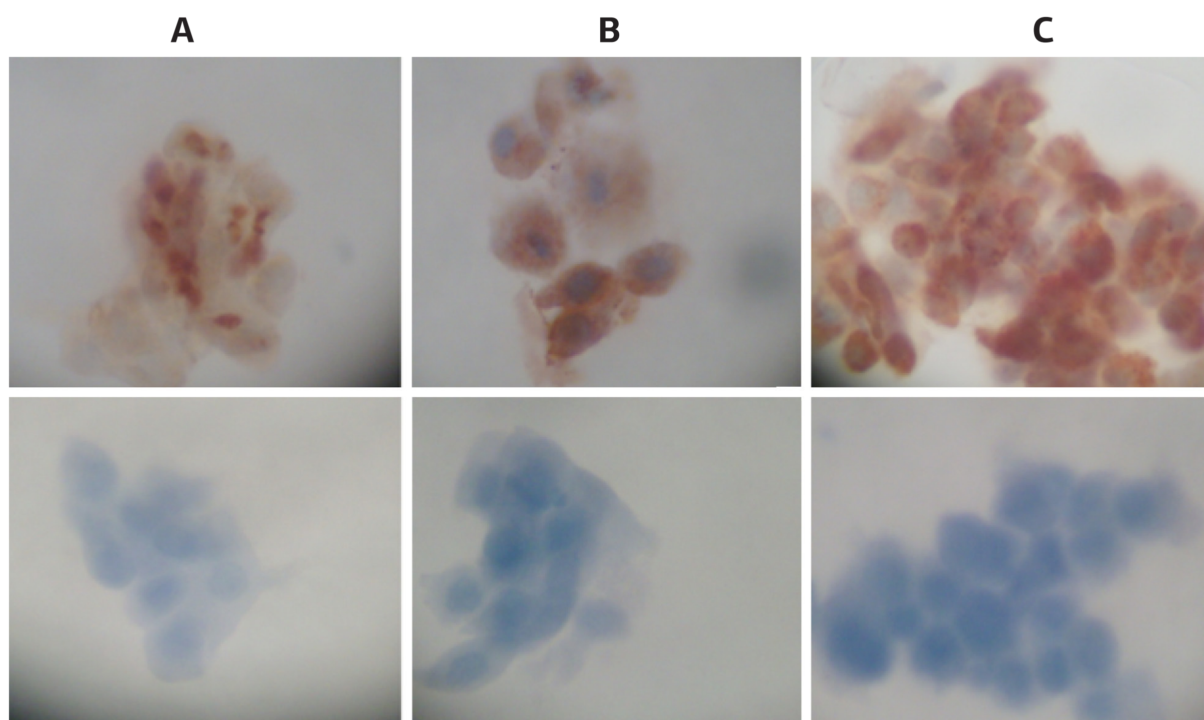

Figure 1. Immunocytochemistry analysis for the markers **A:** p53 expression (nuclear staining pattern). **B:** PTEN expression (cytoplasmic mainly and nuclear staining pattern). **C:** survivin (nuclear predominantly and cytoplasmic staining pattern). Second row: negative expression, original magnification 400×.

was 63 years. All of the patients were informed and consented to the use of these cell samples for research purposes, according to World Medical Association Declaration of Helsinki guidelines. Using a liquid-based cytology assay (LiquiPrep, LGMI, Inc, Melbourne, FL, USA) all of the cell specimens were collected and fixed accordingly. Among them, 38 were obtained from pterygia and the remaining 12 from normal epithelia (control group). The final fixed slides were used for immunocytochemistry (ICC) analysis.

Immunocytochemistry assay

ICC for survivin, p53 and PTEN antigens was carried out on liquid-based fixed slides described above. Anti-survivin mouse monoclonal antibody (clone 12C4, Dako NA Inc, CA, USA; dilution at 1:100), anti-p53 mouse monoclonal antibody (clone DO-7, Dako NA Inc, CA, USA; dilution at 1:40), anti-PTEN mouse monoclonal antibody (clone 6H2.1, Dako NA Inc, CA, USA; dilution at 1:100) were applied properly according to the EN Vision + (Dako NA Inc, CA, USA) assay using an automated staining system (I 6000, Biogenex, USA). This specific assay is based on a soluble, dextran-polymer system preventing an endogenous biotin reaction and increasing the quality of the stained slides. Briefly, the sections were incubated with the primary antibody for 30 min at room temperature and then incubated with the polymer for 30 min. The antigen-antibody reaction was visualized using 3, 3- diaminobenzidine tetrahydrochloride (DAB) as a chromogen substrate. Finally, the slides were slightly counterstained with hematoxylin for 30 s, dehydrated and mounted. According to the manufacturer's guidelines, breast cancer tissue samples previously reported to express these markers were used as positive controls for them. Nuclear staining pattern was acceptable for p53 expression, whereas cytoplasmic mainly and nuclear pattern were both acceptable for PTEN expression. Concerning survivin, nuclear predominantly and cytoplasmic immunostaining was considered as acceptable expression (Figure 1A-C). Digital image analysis was performed to objectively evaluate the corresponding immunostaining intensity levels.

Digital image analysis (DIA) assay

In order to evaluate p53, PTEN and survivin ICC results, in an accurate objective and fast way, DIA was performed by applying a semi-automated system with the following hardware features: Intel Pentium Dual-Core, Digital Camera Sony Cyber-shot (5Mp digital analysis), Microscope Olympus CX-31 and the corresponding software: Windows XP platform/NIS–elements image analysis software, Nikon, Jp 2009. A digitized database was constructed including all the snapshots of the immunostained liquid cytology-based slides. A macro was implemented for evaluating those slides. Measurements of staining intensity values were performed in 5 optical fields per case and at original magnification of 400x. All measurements were performed inside a stable active window focused on isolated or clusters of detected epithelial mainly and stromal cells (Figure 2).

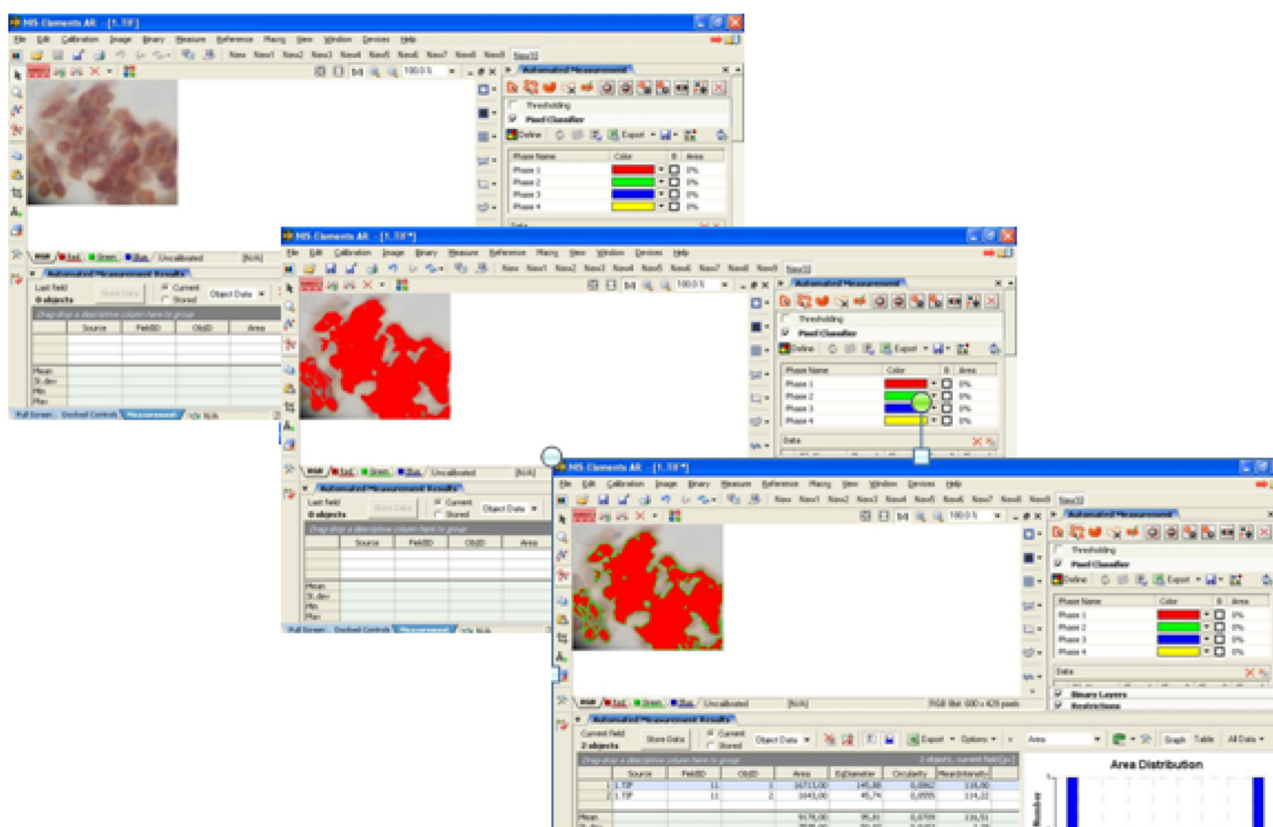

Figure 2. Computerized image analysis of a case of survivin protein expression objective evaluation. Reddish areas demonstrate different expression values (range: 0-255 grey scale immunostaining intensity levels).

Table 1. Total results of the examined markers and also p values

n=50	*p53*			*PTEN*			*Survivin*		
	L	*OE*	*p value*	*L*	*OE*	*p value*	*L*	*OE*	*p value*
Pterygia (n=38)	16	22	0.03	13	25	0.456	8	30	0.019
Type			NS			NS			NS
Flat (n=22)	10	12		9	13		5	17	
Fleshy (n=16)	6	10		4	12		3	13	
Localization			NS			NS			NS
Central (n=18)	10	8		11	7		4	14	
Peripheral (n=20)	8	12		2	18		7	13	
Control (n=12) (normal epithelia)	1	11		4	8		9	3	

L: low expression, OE: overexpression, NS: non significant
p53 mean values (pterygia: 126.4, control: 147.9); PTEN mean values (pterygia: 121.6, control: 139.5); Survivin mean values (pterygia: 113.7, control: 141.7). p53 staining intensity levels (range 0-255). p53/PTEN co-expression (p=0.022).

All numerical data were filed in Microsoft Excel sheets for statistical analysis. Staining intensity levels were obtained in a range of 256 continuous values, including the RGB protocol in grey scale as follows: 0: absolute black (ultimate overexpression), 255: absolute white (loss of expression). Control group values (normal epithelia *vs* pterygia) were obtained also by the corresponding measurements.

Statistics

Associations between PTEN, p53, and survivin protein expression levels and pterygia clinicopathological parameters were performed by applying chi-square and Fischer's tests (SPSS v 20.0 statistical software- Inc Chicago IL, USA). Total results regarding the corresponding grouped measurements for the examined markers and also p values are described in Table 1.

Results

According to previous referred protein analysis mediated by DIA, all of the examined cases demonstrated PTEN/surviving/p53 expression in different immunostaining levels. The majority of the examined pterygia cases overexpressed the markers (p53: 22/38-57.9%, survivin: 30/38-78.9%, and PTEN: 25/38-65.7%, respectively). Interestingly, overall p53/PTEN co-expression was found to be statistically significant (p=0.022). Overexpression of survivin mainly and of p53 was significantly associated to abnormal conjunctiva epithelia (p=0.019, p=0.03, respectively). No statistical significance was observed regarding the age of the examined patients.

Discussion

Based on extensive molecular analyses, tissue stability is mediated by a balance between cellular proliferation and apoptosis [9]. The way that normal conjunctival epithelia transform into pterygium lesion is under investigation. Some studies have shown that the progressive development of the lesion should be a result of an aberrant expression of apoptotic and also anti-apoptotic factors. P53 expression combined with bcl-2 (an inhibitor of apoptosis) and also Bax (a promoter of apoptosis) increased levels have been detected in pterygia [10]. This genetic mechanism is not observed in normal epithelia. In fact, the role of p53 is to prevent the aberrant expression of bcl-2 and to induce the bax protein production. Besides this genetic deregulation, survivin seems to be a crucial molecule in pterygia development. As a member of the apoptosis inhibitors superfamily, suppresses the apoptotic process. Concerning pterygia, elevated gene transcription leads to protein overexpression in them. A study group analyzed the marker at the m-RNA and protein level showing that phosphorylated survivin was detected mainly in the cytoplasm and also in the nucleus [11]. Interestingly, they suggested that the protein could be targeted for the treatment of the lesion by specific siRNA implementation *in vitro* by mixed keratinocyte and pterygium epithelial cells. Similarly, another study showed that the combined p53 and survivin overexpression was correlated with not only primary development of pterygia but also with their recurrence [12]. According to our protein expression results, survivin mainly and p53 increased immunoreactivity was associated to abnormal conjunctiva epithelia. This observation enhances the role of the molecule in the deregulation of the apoptotic mechanism in these epithelia. Furthermore, the molecule seems to overreact with other proteins including cyclooxygenase-2 (COX-2) and the cyclic

AMP response element-binding protein (CREB) at the basis of pterygia rise [13,14]. Additionally to those interactions, survivin demonstrates an indirect relationship with oxidative DNA damage. According to a relative study, oxidative stress potentially leads to a significant activation of survivin expression promoting development of pterygium [15].

The role of p53 is also under consideration in pterygia neoplastic progression. There are controversial data regarding the expression of the marker. Some studies support the idea that, although abnormal expression is observed in primary pterygial epithelia, there was no connection between them and recurrence of the lesion [16,17]. Based on our immunocytological expression results, overall p53/PTEN co-expression was found to be significantly associated to abnormal conjunctiva epithelia. This observation shows the potential active role of PTEN influence in the development of the lesion. There are no gross and solid data regarding the PTEN expression involvement in the pathogenesis of the pterygium, although it is implicated in critical cellular actions. Embryo-histogenetic analyses have demonstrated that PTEN expression regulates significantly the retinal architecture modifying its functionality, and also its downregulation in injured corneal epithelium induces cell migration and wound healing by promoting PI3K/Akt signaling pathway [18-20]. Additionally, experimental studies in biological species, such as mice and Drosophila, proved that PTEN affects cell size, cell proliferation and apoptosis during their eye development [21]. Concerning pterygia development, PTEN overactivation that significantly suppresses signal tranduction to the nucleus could be an indirect mechanism in the apoptotic process. In fact, previous studies analyzing its role in tumor genesis have shown that PTEN-induced downregulation of survivin increased apoptosis in tumor cells, in a reaction reversed by re-expression of recombinant survivin [22,23]. Finally, in our study we implemented a version of exfoliative cytology - liquid based scraping - in order to collect the corresponding specimens and apply immunocytochemistry protocols for the analyzed proteins. This is an easy to use, reliable technique, which has been almost recently performed in similar studies for detecting conjunctival lesions, such as ocular surface squamous neoplasms of the eye [24].

In conclusion, our experimental findings suggest that survivin's overexpression leads to an increased anti-apoptotic activity, playing a central molecular role in the pathogenesis and progression of pterygia. Furthermore, p53 expression is also observed in these lesions, but its impact seems to be low compared to survivin's influence on them. Also, the role of PTEN in the current process is potentially significant providing a suppressor balance to the p53/survivin complex. Additionally, our data support the current growing belief, that pterygia should be regarded as locally expanding neoplastic lesions. Furthermore, their growth affects the normal function of their surrounding tissues, namely the conjuctiva and the cornea, causing chronic irritation and discomfort and most significantly deterioration of vision. Their current surgical treatment carries a low success rate and the use of specific antineoplastic agents may prove the best approach to their treatment.

Conflict of interests

The authors declare no conflict of interests.

References

1. Liu T, Liu Y, Xie L et al. Progress in the pathogenesis of pterygium. Curr Eye Res 2013;38:1191-7.
2. Pajic B, Vastardis I, Rajkovic P et al. A mathematical approach to human pterygium shape. Clin Ophthalmol 2016;10:1343-9.
3. Tan DTH, Tang WY, Liu YP et al. Apoptosis and apoptosis related gene expression in normal conjunctiva and pterygium. Br J Ophthalmol 2000;84:212-6.
4. Dushku N, Reid TW. P53 expression in altered limbal basal cells of pingueculae, pterygia, and limbal tumors. Curr Eye Res 1997;16:1179-92.
5. Guha M, Altieri DC. Survivin as a global target of intrinsic tumor suppression networks. Cell Cycle 2009;8:2708.
6. Mita AC, Mita MM, Nawrocki ST et al. Survivin: key regulator of mitosis and apoptosis and novel target for cancer therapeutics. Clin Cancer Res 2008;14:5000-5.
7. Wang X, Jiang X. PTEN: a default gate-keeping tumor suppressor with a versatile tail. Cell Res 2008;18:807-816.
8. Sakagami K, Chen B, Nusinowitz S et al. PTEN regulates retinal interneuron morphogenesis and synaptic layer formation. Mol Cell Neurosci 2012;49:171-83.
9. Grossman D, McNiff JM, Li F et al. Expression and targeting of the apoptosis inhibitor, survivin, in human melanoma. J Invest Dermatol 1999;113:1076-81.
10. Miyashita T, Reed JC. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell 1995;8:293-9.

11. Xu YX, Zhang LY, Zou DL et al. Differential expression and function of survivin during the progress of pterygium. Invest Ophthalmol Vis Sci 2014;55:8480-7.
12. Zhang LW, Chen BH, Xi XH et al. Survivin and p53 expression in primary and recurrent pterygium in Chinese patients. Int J Ophthalmol 2011;4:388-92.
13. Maxia C, Perra MT, Demurtas P et al. Relationship between the expression of cyclooxygenase-2 and survivin in primary pterygium. Mol Vis 2009;15:458-63.
14. Nubile M, Curcio C, Lanzini M et al. Expression of CREB in primary pterygium and correlation with cyclin D1, ki-67, MMP7, p53, p63, Survivin and Vimentin. Ophthalmic Res 2013;50:99-107.
15. Maxia C, Perra MT, Demurtas P et al. Expression of survivin protein in pterygium and relationship with oxidative DNA damage. J Cell Mol Med 2008;12:2372-80.
16. Weinstein O, Rosenthal G, Zirkin H et al. Overexpression of p53 tumor suppressor gene in pterygia. Eye (Lond) 2002;16:619-21.
17. Liang K, Jiang Z, Ding BQ et al. Expression of cell proliferation and apoptosis biomarkers in pterygia and normal conjunctiva. Mol Vis 2011;17:1687-93.
18. Tsai YY, Chang CC, Chiang CC et al. HPV infection and p53 inactivation in pterygium. Mol Vis 2009;15:1092-7.
19. Cantrup R, Dixit R, Palmesino E et al. Cell-type specific roles for PTEN in establishing a functional retinal architecture. PLoS One 2012;7:e32795.
20. Cao L, Graue-Hernandez EO, Tran V et al. Downregulation of PTEN at corneal wound sites accelerates wound healing through increased cell migration. Invest Ophthalmol Vis Sci 2011;52:2272-8.
21. Huang H, Potter CJ, Tao W et al. PTEN affects cell size, cell proliferation and apoptosis during Drosophila eye development. Development 1999;126:5365-72.
22. Cárdenas-Cantú E1, Zavala J1, Valenzuela J et al. Molecular Basis of Pterygium Development. Semin Ophthalmol 2016;31:567-83.
23. Guha M, Plescia J, Leav I et al. Endogenous tumor suppression mediated by PTEN involves survivin gene silencing. Cancer Res 2009;69:4954-8.
24. Kayat KV, Correa Dantas PE, Felberg S Galvão MA, Saieg MA. Exfoliative Cytology in the Diagnosis of Ocular Surface Squamous Neoplasms. Cornea 2017;36:127-30.