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c-FOS Expression Analysis in Pterygia Cell Spot Arrays

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Abstract. *Background/Aim: Mechanisms of c-FOS activation in the onset and progression of pterygia remain under investigation. This study aimed to comparatively analyze c-FOS proto-oncogene expression levels in neoplastic pterygia and normal epithelia. Materials and Methods: We used a liquid-based cytology assay on thirty (n=30) pterygia cell populations and normal epithelia (n=10) extracted by a smooth scraping of conjunctiva epithelia. Applying a cell spot-based technique, we constructed five (n=5) slides, each containing eight (n=8) cell spots. A modified immune-cytochemistry (ICC) assay for c-FOS protein was used. Additionally, digital image analysis was implemented to calculate c-FOS immunostaining intensity levels. Results: High staining intensity levels of c-FOS were detected in 12/30 (40%), whereas the rest 18/30 (60%) demonstrated moderate expression. c-FOS levels were statistically significantly higher in the pterygia compared to control tissues (p=0.001). c-FOS levels in the pterygia were not associated with the sex of patients (p=0.678), the presence of recurrent lesion (p=0.390) or the location of the lesion (p=0.158). The levels of c-FOS, however, were marginally significantly affected by the morphology of the pterygia (p=0.005). More precisely, the c-FOS levels were significantly higher in pterygia with a fleshy morphology. Conclusion: c-FOS over-expression is observed frequently in pterygia. It seems to be critically involved in the molecular mechanism of the lesion by its over-expression affecting partially their morphological features. Cell spot liquid – based array analysis is an innovative, easy to implement technique for simultaneously evaluating a broad spectrum of molecules in multiple specimens on the same slide.*

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Over-activation of oncogenes critically influences the genomic substrate in pre-malignant neoplastic epithelia transforming them into their malignant cell phenotype. Among oncogene-depended pathways, including those involving transcription factors, the c-FOS/c-JUN complex leads to an aberrant expression of other crucial genes responsible for cell homeostasis (1, 2). Up-regulation of c-FOS and c-JUN proto-oncogenes -due to increased copy numbers (amplification) or intra-genic point mutations- correlates with aggressive biological behavior of malignancies of different tissue origin (3-5). The *FOS* proto-oncogene, also named activator protein-1 (AP-1) transcription factor subunit (*c-FOS*), belongs to a category of extensively analysed genes involved in the onset and progression of solid malignancies. The FOS super family includes c-*FOS, FOSB, FOSL1,* and *FOSL2* genes. *c-FOS* is the human homolog of the retroviral oncogene *v-FOS* (gene locus: 14q24.3). It was first detected and finally cloned in rat fibroblasts as the transforming gene of Finkel–Biskis–Jinkins murine osteogenic sarcoma virus (6). The corresponding gene encodes a 62 kDa protein (380 amino acids), which forms a heterodimer with c-JUN, a transcription factors, resulting in the formation of AP-1 complex. The c-FOS/c-JUN complex influences intracellular signal transduction to the nucleus where each member of the complex interacts

Table I. *Clinicopathological features of the examined pterygia cases and total c-FOS protein expression results.*

Clinicopathological parameters N=40	n (%)	c-FOS		*p*-Value
		H&M expression	L expression	
Pterygia	n=30	30/30 (100%)	0/30 (0%)	
Controls	n=10	1/10 (10%)	9/10 (90%)	**0.001**
Sex				0.678
Male	10/30 (33%)	10/30 (33%)	0/30 (0%)	
Female	20/30 (67%)	20/30 (67%)	0/30 (0%)	
Anatomic location				0.158
Central	16 (53%)	16/30 (53%)	0/30 (0%)	
Peripheral	14 (47%)	14/30 (47%)	0/30 (0%)	
Morphology type				**0.005**
Flat	18 (60%)	18/30 (60%)	0/30 (0%)	
Fleshy	12 (40%)	12/30 (40%)	0/30 (0%)	
Recurrence status				0.390
Relapse	4 (16%)	4/30 (16%)	0/30 (0%)	
Non-relapse	26 (84%)	26/30 (84%)	0/30 (0%)	

c-FOS: Cellular (fibro) proto-oncogene; H/M: Moderate/High over-expression (staining intensity values ≤141/129 (spectrum between 93 and 140/128); L: Low expression (staining intensity values ≤142 (spectrum between 149 and 162). Statistically significant *p*-values are shown in bold.

equally with symmetrical DNA half-sites. Additionally, c-FOS protein is involved in some crucial intracellular functions including tissue differentiation, cell proliferation, cell survival, and tissue homeostasis in response to hypoxia and angiogenesis signals (7, 8).

Concerning pterygium, clinico-pathological studies have shown that it refers to a non-neoplastic, although mimicking, lesion derived from the ocular surface. Degenerative and hyperplastic conjunctival epithelia are the substrate for the lesion. Histo-pathologically, excessive fibroblastic and vascular proliferation lead to an abnormal tissue complex characterized also by chronic inflammatory infiltration (9, 10). Furthermore, the modified tissue invades the cornea leading to specific clinical characteristics including corneal dellen, progressively irregular astigmatism, and potentially severe vision loss (11, 12). In the current experimental study, we focused on c-FOS protein expression in pterygia and normal conjunctiva epithelia by applying a cell spot-based array technique on cytological slides followed by a digital image analysis assay for objectively calculating the corresponding c-FOS protein levels.

Materials and Methods

Study design and patients. A total of thirty (n=30) patients diagnosed with pterygium conjunctival abnormality were enrolled. The mean age of the corresponding patients was 67 years. Concerning the sex, twenty females and ten males were included in the study. This study was performed in the Department of Ophthalmology and approved by the Research Ethics Committee of the Medical School, University of Patras, Rio-Patra, Greece (Decision reference number: 310/06.07.2017), according to the World Medical Association and the Declaration of Helsinki guidelines.

Cell substrates. Thirty (n=30) cell specimens were used by applying a smooth scraping on the corresponding epithelial surface of the pterygium. Scraping depth was evaluated in the next step. Implementing a liquid-based cytology assay (Cell Solutions, Menarini, Florence, Italy) all cell populations were properly collected and fixed. All patients declared no HPV infection diagnosed using PCR. The same process was also applied in normal conjunctiva epithelia (n=10; control group). In 12 of the examined cases, pterygium was recognized in only one eye. Eighteen (n=18) cases had bilateral lesion. In the control group (normal appearing) epithelia were obtained from the non-pterygium areas of the corresponding eyes. Histopathological features were confirmed by biopsy. Two ophthalmologists categorized pterygia, according to their clinical features and macroscopic morphology. The final agreement was 100%, although for one case there was a slight disagreement. Concerning the last case, the two pathologists reached a final agreement based on the area and depth of the lesion. All patients subjected to bare sclera technique for pterygium excision by the same surgeon. Clinicopathological parameters of the examined cases are listed in Table I.

Cell spot array slide construction. Five (n=5) multi-spot slides were constructed. Each contained eight (n=8) cell spots (spot diam: ~0.5 cm). Two columns with four spot rows were recognized on the surface of the corresponding

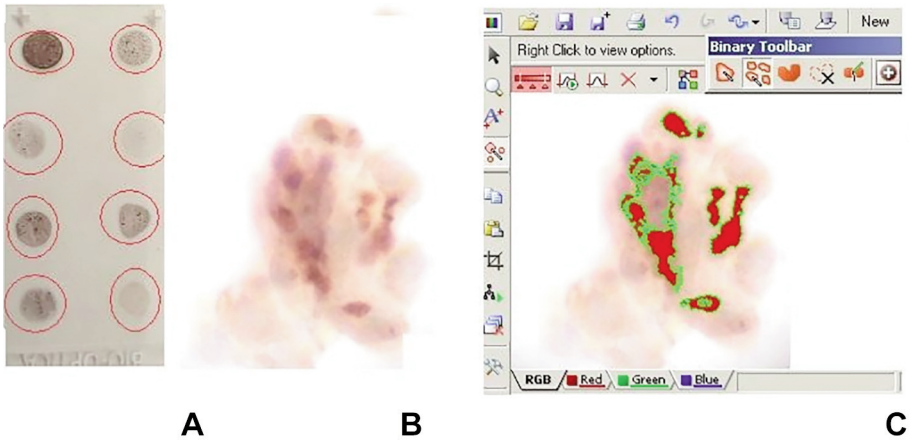

Figure 1. *c-FOS protein expression levels in pterygia fibro-endothelial cells A) Cell spot array. Note 8 spots of liquid-based cytological specimens obtained by scrapping, B) c-FOS dense expression [dark brown nuclear mainly, cytoplasmic staining pattern produced using 3-3,diaminobenzidinetetrahydrocloride (DAB) chromogen, original magnification 400×], C) Digital image analysis on a pterygia cell spot stained using a c-FOS antibody. In this progressive accurate and objective measurement process, reddish areas demonstrate different expression values, whereas green lines encircle specific areas of interest (range=0-255 continuous grey scale immunostaining intensity levels).*

slides. We detected microscopically all (n=40) examined pterygia and control cases as spots on the surface of the slides (confirmation of the adequacy of the examined specimens) (Figure 1A).

Antibodies and immunocytochemistry assay (ICC). Ready-to-use mouse monoclonal anti-c-FOS (clone CF2, Novocastra, Leica Biosystems, Newcastle, UK) antibody at 1:40 dilution in Tris-buffered saline (TBS) with 1% bovine serum albumen (BSA) was applied in the corresponding cell spot array slides. The ICC process was performed using the En Vision protocol (DAKO, Glostrup, Denmark) on an automated IHC-ICC staining system (I 6000-Biogenex, Fremont, CA, USA). The ICC protocol was applied in a similar way and modified as described in a previous study by our study group (13). Nuclear predominantly but also peri-nuclear staining pattern was acceptable for specificity of the examined protein according to the instructions of the manufacturer (Figure 1B).

Digital image analysis (DIA). c-FOS protein expression levels were measured in a quantitative way as staining intensity levels (densitometry evaluation) in the stained pterygia and normal cell spots. We performed DIA using a semi-automated system (Microscope CX-31, Olympus, Melville, NY, USA; Digital camera, Sony, Tokyo, Japan; Windows XP/NIS-Elements Software AR v3.0, Nikon Corp, Tokyo, Japan). According to the protocol, specific areas of interest per cell spot were detected (5 optical fields at ×400 magnification) and filed in a digital database as snapshots. Staining intensity values were extracted by implementing a specific macro (nuclear/peri-nuclear/cytoplasmic staining pattern, according to manufacturer's datasheet for monoclonal mouse anti-c-FOS antibody). A specific algorithm was constructed for normal (control) cell spots and pterygia immunostained cells. A broad spectrum of continuous grey scale values (0-255) at the RedGreenBlue (RGB) analysis was available for discriminating different protein expression levels (Figure 1C). c-FOS staining intensity values decreasing to 0 show progressive over-expression of the marker, whereas values increasing to 255 present a progressive loss of staining intensity of the examined marker. All results and DIA values are presented in Table I.

Statistical analysis. The software package IBM SPSS v25 (SPSS Inc, Chicago, IL, USA) was implemented for statistical analysis. Associations between variables were extracted by applying Pearson Chi-Square (χ^2) test and Fisher's exact test. Correlation analysis for detecting associations between staining intensity values and variables was performed using Spearman Rank test. Two-tailed *p*-values ≤0.005 were considered statistically significant. ICC results and *p*-values are described in Table I.

Results

Total protein analysis based on DIA results showed that all examined cases demonstrated different c-FOS immunostaining levels. Nuclear/peri-nuclear/cytoplasmic focal or diffuse staining immunoreactivity in a variety of small fibrous and epithelial/endothelial cell fragments was detected. High (dense) staining intensity values were detected in 12/30 (40%), whereas the rest 18/30 (60%) were characterized by moderate

expression. The overall c-FOS levels were statistically significantly higher in the pterygia compared to the control tissues (p=0.001). In the analyzed pterygia c-FOS levels did not seem to corelate to the sex of the patients (p=0.678), with the presence of recurrent lesion (p=0.390) or the location of the pterygia (p=0.158). The levels of c-FOS, however, were marginally affected by the morphology of the pterygia (p=0.005). In particular, c-FOS levels were significantly higher in the pterygia with a fleshy morphology.

Discussion

Concerning its frequency, pterygium is detected in adults -as they age- especially in specific locations (14, 15). Etiopathogenetic factors include persistent inflammatory agents such as chronic exposure to high levels of ultraviolet (UV) radiation, viral infections combined or not with a level of genetic predisposition (16). Referring to UV chronic exposure, elevated oxidative-based DNA damage triggers a central intra-cellular biochemical mechanism involved in pterygia onset. In this case, epithelial micro-environment transformation combined or not with ischemic tissue injury and progenitor cell tropism induce pterygium's onset and progression (17). A variety of gene and protein analyses have reported that lesion development and progression are associated with deregulation of critical genes involved in cell proliferation, angiogenesis, signal transduction to the nucleus, and apoptosis (18, 19). Specifically, vascular endothelial growth factor (VEGF) modifies vascular microenvironment in pterygia due to its neo-angiogenic activity (20). Additionally, other molecules, including angiogenic/pro-inflammatory cytokines, matrix metalloproteinases (MMPs) and their natural tissue inhibitors (TIMPs), are also involved in the progression of the neoplastic-like pterygia phenotype (21-23). Interestingly, activation of signal transduction pathways mediated by epidermal growth factor receptor (EGFR) and keratinocyte growth factor receptor (KGFR) over-expression represent critical UV-dependent molecular mechanisms (24-26). Besides these receptors, mTOR signaling over-activation seems to induce an increased profibrotic activity in TGF-β1-induced myofibroblast differentiation. A study group suggested that an anti-m TOR inhibitor (rapamycin) should be applied in pterygia demonstrating TGF-β1-induced myofibroblastic responses (27). Additionally, deregulation of mouse double minute 2 (MDM2)/p53 protein complex has been also observed in pterygia onset and progression. A study showed that a specific inhibitor (nutlin) targeting MDM2 leads to disruption of the MDM2/p53 pathway (28).

In the current experimental study, we analyzed c-FOS protein expression in a series of pterygia and corresponding normal epithelia (control group) by applying a cell spot-based array on cytological slides combined with a digital image analysis process for measuring objectively the corresponding protein levels. In fact, cell specimens provide a partially limited but vital substrate for a broad range of molecular analyses. Slight tissue scraping of the surface or intra-operative neoplasms is an easy-to-use method that maintains morphology and secures integrity of the obtained cells or small fragment laid on the slides. In contrast to tissue micro-sectioning procedure that leads to a partial loss of nucleus, cytological methods prevent nuclear volume integrity (29). A variety of protein markers have already analyzed by applying a combination of liquid-based cytology, ICC, and DIA protocols (30, 31). In the current research, we improved and enriched this technique by constructing cell spot arrays on the corresponding slides. This represents the tissue microarray analogue for handling many cytological specimens in only one slide. Additionally, we implemented a specific algorithm for measuring c-FOS staining intensity levels on pterygia and normal cells objectively and accurately (32, 33).

ICC analysis demonstrated significant expression of c-FOS in pterygia compared to control group, and an interesting, selective over-expression associated to fleshy morphology. In conjunction to our results, two previous molecular and protein expression studies reported increased c-FOS gene expression correlated with AP-1 over-activation in UVB-exposed keratinocytes (34, 35). Induction of combined c-FOS/c-JUN protein complex -at the mRNA level *in vitro*- have been also detected in molecular studies that revealed the crucial role of UVB in the corresponding epithelia (36-38). Furthermore, aberrant expression of microRNAs (miRs) seems to be implicated in the development and progression of pterygia. A study group analysed the differential expression of long non-coding RNAs (lncRNAs), miRs, and messenger RNAs (mRNAs) in a series of pterygia. They reported a significant deregulation of lncRNA SNHG1/miR-766-3p/FOS in the corresponding tissues that are involved in an unexplored mechanism affecting pterygia onset (39). In the current study, all of the examined patients demonstrated no history of HPV infection. Concerning the involvement of HPV and other persistent viral infections, including Epstein Barr, in pterygia pathogenesis there are controversial experimental and epidemiological results (40-43).

In conclusion, our experimental findings suggest that c-FOS oncogene overexpression is a frequent and crucial intracellular event in pterygia playing potentially a central molecular role in the progression of the lesion. Cell spot-based array analysis on liquid cytology fixed slides seems to be an easy-to-use technique for analyzing a broad variety of molecules in multiple specimens on the same slide by applying different ICC assays.

Conflicts of Interest

The Authors have no conflicts of interest to declare in relation to this study.

Authors' Contributions

S. Mastronikolis, E. Tsiambas: design of the study, manuscript writing, C.D. Georgakopoulos: conduct of the study, O.E. Makri, M. Pagkalou: collection and management of data, V.K. Thomopoulou: analysis/data interpretation, D. Peschos, V. Ragos: academic advisor. All Authors read and approved the final manuscript.

References

1 Hanahan D and Weinberg RA: Hallmarks of cancer: the next generation. Cell *144(5)*: 646-674, 2011. PMID: 21376230. DOI: 10.1016/j.cell.2011.02.013

2 Ali J, Sabiha B, Jan HU, Haider SA, Khan AA and Ali SS: Genetic etiology of oral cancer. Oral Oncol *70*: 23-28, 2017. PMID: 28622887. DOI: 10.1016/j.oraloncology.2017.05.004

3 Mishra A, Bharti AC, Saluja D and Das BC: Transactivation and expression patterns of Jun and Fos/AP-1 super-family proteins in human oral cancer. Int J Cancer *126(4)*: 819-829, 2010. PMID: 19653276. DOI: 10.1002/ijc.24807

4 Turatti E, da Costa Neves A, de Magalhães MH and de Sousa SO: Assessment of c-Jun, c-Fos and cyclin D1 in premalignant and malignant oral lesions. J Oral Sci *47(2)*: 71-76, 2005. PMID: 16050486. DOI: 10.2334/josnusd.47.71

5 Gupta S, Kumar P, Kaur H, Sharma N, Saluja D, Bharti AC and Das BC: Selective participation of c-Jun with Fra-2/c-Fos promotes aggressive tumor phenotypes and poor prognosis in tongue cancer. Sci Rep *5*: 16811, 2015. PMID: 26581505. DOI: 10.1038/srep16811

6 Curran T, Peters G, Van Beveren C, Teich NM and Verma IM: FBJ murine osteosarcoma virus: identification and molecular cloning of biologically active proviral DNA. J Virol *44(2)*: 674-682, 1982. PMID: 6292525. DOI: 10.1128/JVI.44.2.674-682.1982

7 Angel P and Karin M: The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. Biochim Biophys Acta *1072(2-3)*: 129-157, 1991. PMID: 1751545. DOI: 10.1016/0304-419x(91)90011-9

8 Adler V, Polotskaya A, Wagner F and Kraft AS: Affinity-purified c-Jun amino-terminal protein kinase requires serine/threonine phosphorylation for activity. J Biol Chem *267(24)*: 17001-17005, 1992. PMID: 1324919.

9 Bradley JC, Yang W, Bradley RH, Reid TW and Schwab IR: The science of pterygia. Br J Ophthalmol *94(7)*: 815-820, 2010. PMID: 19515643. DOI: 10.1136/bjo.2008.151852

10 Detorakis ET and Spandidos DA: Pathogenetic mechanisms and treatment options for ophthalmic pterygium: trends and perspectives (Review). Int J Mol Med *23(4)*: 439-447, 2009. PMID: 19288018. DOI: 10.3892/ijmm_00000149

11 Liu T, Liu Y, Xie L, He X and Bai J: Progress in the pathogenesis of pterygium. Curr Eye Res *38(12)*: 1191-1197, 2013. PMID: 24047084. DOI: 10.3109/02713683.2013.823212

12 Janson BJ and Sikder S: Surgical management of pterygium. Ocul Surf *12(2)*: 112-119, 2014. PMID: 24725323. DOI: 10.1016/j.jtos.2014.01.001

13 Tziakou P, Theodoropoulos G, Tsiambas E, Zizi-Sermpetzoglou A, Peschos D, Mastronikoli S, Thomopoulou G, Thymara E, Kavantzas N and Lazaris AC: Impact of PD-L1 protein expression on renal cell carcinoma histo-differentiation. Anticancer Res *41(8)*: 3809-3813, 2021. PMID: 34281840. DOI: 10.21873/anticanres.15173

14 Uy HS, Reyes JM, Flores JD and Lim-Bon-Siong R: Comparison of fibrin glue and sutures for attaching conjunctival autografts after pterygium excision. Ophthalmology *112(4)*: 667-671, 2005. PMID: 15808260. DOI: 10.1016/j.ophtha.2004.08.028

15 Rezvan F, Khabazkhoob M, Hooshmand E, Yekta A, Saatchi M and Hashemi H: Prevalence and risk factors of pterygium: a systematic review and meta-analysis. Surv Ophthalmol *63(5)*: 719-735, 2018. PMID: 29551597. DOI: 10.1016/j.survophthal.2018.03.001

16 Cárdenas-Cantú E, Zavala J, Valenzuela J and Valdez-García JE: Molecular basis of pterygium development. Semin Ophthalmol *31(6)*: 567-583, 2016. PMID: 25415268. DOI: 10.3109/08820538.2014.971822

17 Kim KW, Ha HS and Kim JC: Ischemic tissue injury and progenitor cell tropism: significant contributors to the pathogenesis of pterygium. Histol Histopathol *30(3)*: 311-320, 2015. PMID: 25314135. DOI: 10.14670/HH-30.311

18 Karukonda SR, Thompson HW, Beuerman RW, Lam DS, Wilson R, Chew SJ and Steinemann TL: Cell cycle kinetics in pterygium at three latitudes. Br J Ophthalmol *79(4)*: 313-317, 1995. PMID: 7742273. DOI: 10.1136/bjo.79.4.313

19 Tan DT, Tang WY, Liu YP, Goh HS and Smith DR: Apoptosis and apoptosis related gene expression in normal conjunctiva and pterygium. Br J Ophthalmol *84(2)*: 212-216, 2000. PMID: 10655200. DOI: 10.1136/bjo.84.2.212

20 Folkman J and Klagsbrun M: Angiogenic factors. Science *235(4787)*: 442-447, 1987. PMID: 2432664. DOI: 10.1126/science.2432664

21 Di Girolamo N, Coroneo M and 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 *167(2)*: 489-503, 2005. PMID: 16049334. DOI: 10.1016/S0002-9440(10)62992-6

22 Di Girolamo N, Coroneo MT and 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 *44(11)*: 4705-4714, 2003. PMID: 14578390. DOI: 10.1167/iovs.03-0356

23 Di Girolamo N, McCluskey P, Lloyd A, Coroneo MT and Wakefield D: Expression of MMPs and TIMPs in human pterygia and cultured pterygium epithelial cells. Invest Ophthalmol Vis Sci *41(3)*: 671-679, 2000. PMID: 10711680.

24 Nolan TM, DiGirolamo N, Sachdev NH, Hampartzoumian T, Coroneo MT and Wakefield D: The role of ultraviolet irradiation and heparin-binding epidermal growth factor-like growth factor in the pathogenesis of pterygium. Am J Pathol *162(2)*: 567-574, 2003. PMID: 12547714. DOI: 10.1016/S0002-9440(10)63850-3

25 Blaudschun R, Brenneisen P, Wlaschek M, Meewes C and Scharffetter-Kochanek K: The first peak of the UVB irradiation-dependent biphasic induction of vascular endothelial growth factor (VEGF) is due to phosphorylation of the epidermal growth factor receptor and independent of autocrine transforming growth factor alpha. FEBS Lett *474(2-3)*: 195-200, 2000. PMID: 10838084. DOI: 10.1016/s0014-5793(00)01605-7

26 Marchese C, Maresca V, Cardinali G, Belleudi F, Ceccarelli S, Bellocci M, Frati L, Torrisi MR and Picardo M: UVB-induced activation and internalization of keratinocyte growth factor receptor. Oncogene *22(16)*: 2422-2431, 2003. PMID: 12717419. DOI: 10.1038/sj.onc.1206301

27 Kim SW, Kim HI, Thapa B, Nuwormegbe S and Lee K: Critical role of mTORC2-Akt signaling in TGF-β1-induced myofibroblast differentiation of human pterygium fibroblasts. Invest Ophthalmol Vis Sci *60(1)*: 82-92, 2019. PMID: 30640966. DOI: 10.1167/iovs.18-25376

28 Cao D, Ng TK, Yip YWY, Young AL, Pang CP, Chu WK and Jhanji V: p53 inhibition by MDM2 in human pterygium. Exp Eye Res *175*: 142-147, 2018. PMID: 29932882. DOI: 10.1016/j.exer.2018.06.021

29 Tsiambas E, Karameris A, Lygeros M, Athanasiou AE, Salemis NS, Gourgiotis S, Ragkos V, Metaxas GE, Vilaras G and Patsouris E: Gene numerical imbalances in cytological specimens based on fluorescence/chromogenic in situ hybridization analysis. J BUON *17(3)*: 593-599, 2012. PMID: 23033306.

30 Kayat KV, Correa Dantas PE, Felberg S, Galvão MA and Saieg MA: Exfoliative Cytology in the diagnosis of ocular surface squamous neoplasms. Cornea *36(1)*: 127-130, 2017. PMID: 27661075. DOI: 10.1097/ICO.0000000000001027

31 Konstantopoulou K, Tsiambas E, Baliou E, Lazaris AC, Kavantzas N, Karameris A, Fotiades PP, Asproudis I, Ragos V, Vilaras G, Patsouris E, Konstantopoulou K, Tsiambas E, Baliou E, Lazaris AC, Kavantzas N, Karameris A, Fotiades PP, Asproudis I, Ragos V, Vilaras G and Patsouris E: Deregulation of p53/survivin apoptotic markers correlated to PTEN expression in pterygium neoplastic cells. J BUON *23(3)*: 826-831, 2018. PMID: 30003758.

32 Chrysovergis A, Papanikolaou VS, Tsiambas E, Ragos V, Peschos D and Kyrodimos E: Digital analysis of BCL2 expression in laryngeal squamous cell carcinoma. Anticancer Res *39(3)*: 1253-1257, 2019. PMID: 30842155. DOI: 10.21873/anticanres.13235

33 Tsiambas E, Riziotis C, Mastronikolis NS, Peschos D, Mortakis A, Kyroysis G, Mastronikolis SN, Batistatou A, Lazaris AC, Patsouris E and Ragos V: Comparative $p16^{IKN4A}$ expression in laryngeal carcinoma and cervical cancer precursors: a real-time grid-based immunocytochemistry analysis. Anticancer Res *38(10)*: 5805-5810, 2018. PMID: 30275203. DOI: 10.21873/anticanres.12920

34 Gonzales M and Bowden GT: The role of PI 3-kinase in the UVB-induced expression of c-fos. Oncogene *21(17)*: 2721-2728, 2002. PMID: 11965545. DOI: 10.1038/sj.onc.1205366

35 Chen W and Bowden GT: Activation of p38 MAP kinase and ERK are required for ultraviolet-B induced c-fos gene expression in human keratinocytes. Oncogene *18(52)*: 7469-7476, 1999. PMID: 10602506. DOI: 10.1038/sj.onc.1203210

36 Fisher GJ, Talwar HS, Lin J, Lin P, McPhillips F, Wang Z, Li X, Wan Y, Kang S and Voorhees JJ: Retinoic acid inhibits induction of c-Jun protein by ultraviolet radiation that occurs subsequent to activation of mitogen-activated protein kinase pathways in human skin in vivo. J Clin Invest *101(6)*: 1432-1440, 1998. PMID: 9502786. DOI: 10.1172/JCI2153

37 Wan YS, Wang ZQ, Voorhees J and Fisher G: EGF receptor crosstalks with cytokine receptors leading to the activation of c-Jun kinase in response to UV irradiation in human keratinocytes. Cell Signal *13(2)*: 139-144, 2001. PMID: 11257459. DOI: 10.1016/s0898-6568(00)00146-7

38 Sesto A, Navarro M, Burslem F and Jorcano JL: Analysis of the ultraviolet B response in primary human keratinocytes using oligonucleotide microarrays. Proc Natl Acad Sci U.S.A. *99(5)*: 2965-2970, 2002. PMID: 11867738. DOI: 10.1073/pnas.052678999

39 Xu N, Cui Y, Dong J and Huang L: Exploring the molecular mechanisms of pterygium by constructing lncRNA-miRNA-mRNA regulatory network. Invest Ophthalmol Vis Sci *61(8)*: 12, 2020. PMID: 32645133. DOI: 10.1167/iovs.61.8.12

40 Uthaithammarat L, Kasetsuwan N, Chongpison Y, Kasetsuwan P, Reinprayoon U, Nilyanimit P and Poovorawan Y: Lack of HPV in pterygium with no evidence of autoinoculation and the role of cytokines in pterygium with dry eye. Sci Rep *11(1)*: 2842, 2021. PMID: 33531557. DOI: 10.1038/s41598-021-82114-6

41 Chalkia AK, Bontzos G, Spandidos DA and Detorakis ET: Human papillomavirus infection and ocular surface disease (Review). Int J Oncol *54(5)*: 1503-1510, 2019. PMID: 30896784. DOI: 10.3892/ijo.2019.4755

42 Otlu B, Emre S, Turkcuoglu P, Doganay S and Durmaz R: Investigation of human papillomavirus and Epstein-Barr virus DNAs in pterygium tissue. Eur J Ophthalmol *19(2)*: 175-179, 2009. PMID: 19253231. DOI: 10.1177/112067210901900201

43 Chong PP, Tung CH, Rahman NA, Yajima M, Chin FW, Yeng CL, Go ES, Chan CM, Yawata N and Yamamoto N: Prevalence and viral load of oncogenic human papillomavirus (HPV) in pterygia in multi-ethnic patients in the Malay Peninsula. Acta Ophthalmol *92(7)*: e569-e579, 2014. PMID: 25043991. DOI: 10.1111/aos.12427

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