·Basic Research·

Expression of microRNAs in fibroblast of pterygium

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Abstract

• **AIM: To screen microRNAs (miRNAs) and set up target miRNAs in pterygium.**

• **METHODS: Primary fibroblasts were isolated from pterygium and Tenon´s capsule and cultured. Immunocytochemical analysis and Western blotting were performed to confirm the culture of fibroblasts. In all, 1733 miRNAs were screened in the first step by using GeneChip ® miRNA3.0 Array. Specific miRNAs involved in the pathogenesis of pterygium were subsequently determined using the following criteria: 1) high reproducibility in a repetitive test; 2) base log value of >7.0 for both control and pterygial fibroblasts; and 3) log ratio of >1.0 between pterygial fibroblasts and control fibroblasts.**

• **RESULTS: Primary screening showed that 887/1733 miRNAs were up –regulated and 846/1733 miRNAs were down –regulated in pterygial fibroblasts compared with those in control fibroblasts. Of the 1733 miRNAs screened, 4 miRNAs, namely, miRNA–143a–3p, miRNA–181a–2–3p, miRNA–377–5p and miRNA–411a–5p, met the above–mentioned criteria. Primary screening showed that these 4 miRNAs were up –regulated in pterygial fibroblasts compared with control fibroblasts and that miRNA –143a –3p had the highest mean ratio compared with the miRNAs in control fibroblasts.**

• **CONCLUSION: miRNA –143a –3p, miRNA –181a –2 –3p, miRNA–377–5p and miRNA–411a–5p are up–regulated in pterygial fibroblasts compared with control fibroblasts, suggesting their involvement in the pathogenesis of pterygium.**

• **KEYWORDS:** microRNA; pterygium; fibroblast

INTRODUCTION

Conjunctival or subconjunctival hyperplasia is a complication of ocular surgery (including strabismus, pterygium, glaucoma, and retinal surgery) and represents the primary pathogenesis of disorders such as pterygium and pinguecula [1-2]. Pterygium is a wing-shaped fibrovascular lesion of the ocular surface that is characterized by extensive chronic inflammation, cell proliferation, connective tissue remodeling, and angiogenesis [3]. Fibroblast activation by transforming growth factor-β plays a key role in the pathogenesis of pterygium[4].

MicroRNAs (miRNAs) are small, non-coding RNAs that regulate gene expression usually by repressing the translation of mRNAs through complementary base pairing [5]. miRNA expression is associated with the pathogenesis of various eye disorders, including cataract and pterygium [6-7]. Recent studies suggest that miRNA-145 levels decrease with an increase in the severity of pterygium and that miRNA-145 expression is negatively correlated with pterygium extension and vascularity [8]. Engelsvold *et al* [9] observed a concerted down-regulation of 4 miRNAs belonging to miRNA-200 family (miRNA-200a, miRNA-200b, miRNA-429 and miRNA-200c/miRNA-141) in pterygial tissues compared with those in the normal conjunctiva. These miRNAs regulate epithelial-mesenchymal transition, suggesting that they are involved in the pathogenesis of pterygium. Differential expression of miRNA-145 and miRNAs belonging to the miRNA-200 family in pterygium suggests that additional miRNAs are involved in its pathogenesis. Therefore, we examined the expression of miRNAs in pterygial fibroblasts to determine other miRNAs involved in the pathogenesis of pterygium.

MATERIALS AND METHODS

This study was approved by the Institutional Review Board (IRB) of Kim's Eye Hospital. All participants included in this study provided written informed consent, and the consent form was approved by the IRB. All procedures used in this study conformed to the guidelines mentioned in the Declaration of Helsinki.

Primary Fibroblast Culture Pterygium samples were obtained from 5 patients with primary pterygium by performing elective surgery. In addition, normal nasal side
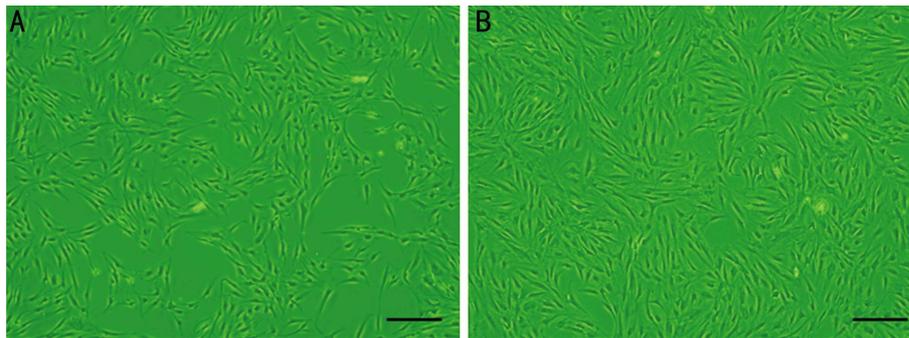
Figure 1 Histological analysis Fibroblasts cultured from pterygial tissue (A) and Tenon's capsule tissue (B). Scale bar: 100 μm.

Tenon's capsule tissues were obtained from 5 controls by performing elective strabismus procedures. All the patients were asked to not use eye drops containing steroids, nonsteroidal anti-inflammatory drugs, and antibiotics.

The conjunctival tissue of pterygium was removed to isolate pure subconjunctival fibroblasts. The isolated fibroblasts (passage 2; density, 2×10^6 cells/p100 dish) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 50 g/mL penicillin, and 50 g/mL streptomycin at 37℃ in a humidified atmosphere containing 5% CO_2(Figure 1).

Immunocytochemical analysis and Western blotting were performed to confirm the culture of fibroblasts. Fibroblasts attached to the chamber slides were washed and fixed with 4% (v/v) formaldehyde for 5min at room temperature. The cells were then permeabilized using 0.05% (v/v) Triton X-100 in phosphate-buffered saline (PBS) for 5min. Next, the cells were incubated overnight at 4℃ with an appropriate primary antibody against vimentin (dilution, 1:100 in PBS; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The cells were then incubated in the dark with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG secondary antibody (dilution, 1:100 in PBS; Vector Laboratories, Peterborough, UK) for 2h at room temperature. Finally, the cells were washed 3 times with PBS and were examined under an immunofluorescence microscope (IX71 instrument; Olympus, Japan). Fibroblasts incubated with normal-buffered serum without the anti-vimentin primary antibody were used as negative controls.

Fibroblasts were washed twice with ice-cold PBS and lysed in cold $1\times$ cell lysis buffer containing 20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L Na_2EDTA, 1 mmol/L EGTA, 1 mmol/L PMSF, 1 μg/mL leupeptin, 1 mmol/L Na_3VO_4, 2.5 mmol/L sodium pyrophosphate, and 1 mmol/L glycerophosphate (Cell Signaling Technology, MA, USA) by sonication on ice for 10min. Protein concentrations in the cell lysates were determined using Bradford reagent (Sigma-Aldrich, Germany). Proteins in the cell lysates were resolved using an appropriate percentage of sodium dodecyl sulfate-polyacrylamide gel and were transferred onto a nitrocellulose membrane. The membrane was incubated overnight with the primary antibody against vimentin at 4℃. After washing, the membrane was incubated with HRP-conjugated sheep anti-mouse IgG secondary antibody (Amersham Pharmacia Biotech Ltd., UK) for 1h at room temperature. Protein signals were detected using an enhanced chemiluminescence (ECL) western blot detection reagent (Amersham Pharmacia Biotech Ltd., UK).

Screening of miRNAs in Fibroblasts

Sample preparation Total RNA was extracted from fibroblasts by using TRI Reagent ® (MRC, OH, USA), according to the manufacturer's protocol. After homogenization, 1 mL of the solution was transferred to a 1.5 mL Eppendorf tube and was centrifuged at 12 000 g× for 10min at 4℃ to remove insoluble material. Supernatant containing the RNA was collected, mixed with 0.2 mL chloroform, and centrifuged at 12 000 g× for 15min at 4℃. Next, RNA in the aqueous phase was transferred to a new tube, was precipitated using 0.5 mL isopropyl alcohol, and was recovered by centrifugation at 12 000 g× for 10min at 4℃. RNA pellet obtained was washed briefly with 1 mL 75% ethanol and was centrifuged at 7500 g× for 5min at 4℃. The RNA pellet was dissolved in nuclease-free water, and quality and quantity of RNA were assessed using Agilent 2100 Bioanalyzer. miRNA expression was assessed using GeneChip ® miRNA 3.0 Array (miRBase Version 17; Affymetrix, Santa Clara, CA, USA), which contains approximately 1733 mature human miRNAs.

Microarray Biotin-3'-labeled DNA was prepared from 1000 ng total RNA by using a standard protocol (Expression Analysis Technical Manual, 2001; Affymetrix). This biotin-3'-labeled DNA was hybridized on GeneChip ® miRNA3.0 Arrays for 16-18h at 48℃. The gene chips were washed and stained in GeneChip ® Fluidics Station 450 (Affymetrix, Santa Clara, CA, USA) and were scanned using GeneChip ® Scanner 3000 7G (Affymetrix, Santa Clara, CA, USA). Data obtained were analyzed by performing RMA and DABG analysis using Affymetrix default settings and were normalized by global scaling. Normalized log transformed intensity values were analyzed using Expression Console software v1.3 (Affymetrix, Santa Clara, CA, USA).
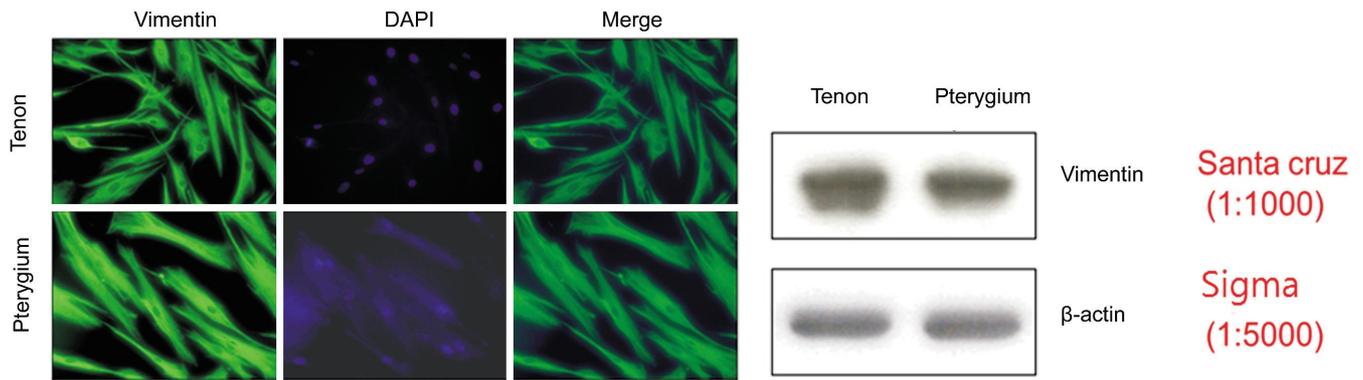
Figure 2 Confocal immunofluorescence microscopy and Western blotting Immunofluorescence analysis and Western blotting detected vimentin in both pterygial and control fibroblasts.

Fold-change filters included the requirement that miRNAs be at least 200% of controls for up-regulated miRNAs and lower than 50% of controls for down-regulated miRNAs. Variation in miRNA expression was analyzed for predicting target miRNAs. Target miRNAs were predicted using TargetScan version 6.2 (http://www.targetscan.org/), with a context score percentile of >90 for determining differentially expressed miRNAs. Hierarchical clustering required clustered groups that behave similarly across experiments using GeneSpring GX 12.6 (Agilent Technologies, CA, USA). Clustering algorithm used Euclidean distance with average linkage.

Secondary miRNA Analysis miRNAs identified during primary screening were analyzed further to determine target miRNAs. Target miRNAs were selected based on the following criteria: 1) high reproducibility in a repetitive test; 2) base log value of >7.0 for both control and pterygial fibroblasts; 3) log ratio of >1.0 between pterygial fibroblasts and control fibroblasts. High reproducibility was defined as the constant expression either upregulation or downregulation.

Total RNA was extracted from cultured fibroblasts, and cDNA was synthesized using first-strand cDNA synthesis kit (GE Healthcare). Primers 143-3p (UGAGAUGAAGCACUG UAGCUC), 181a-2-3p (ACCACUGACCGUUGACUGUAC C), 377-5p (AGAGGUUGCCCUUGGUGAAUUC), and 411a-5p (UAGUAGACCGUAUAGCGUACG) and SYBR Green-based real-time PCR system (MyIQ, Bio-Rad) were used to compare the expression of mature miRNAs in pterygial and control fibroblasts. Data were analyzed using SPSS ver. 15.0 (SPSS Inc., Chicago, IL, USA). Mann-Whitney U test was used to differentiate miRNA levels in the 2 groups. Differences were considered significant at P-values of <0.05.

RESULTS
The mean ages of patients with pterygium (2 men and 3 women) and controls (2 men and 3 women) were 62.5y (range, 49-75y) and 63.5y (range, 52-74y), respectively (P=0.675).

Primary Fibroblast Culture Under the immunohistochemistry, single images of the sections of
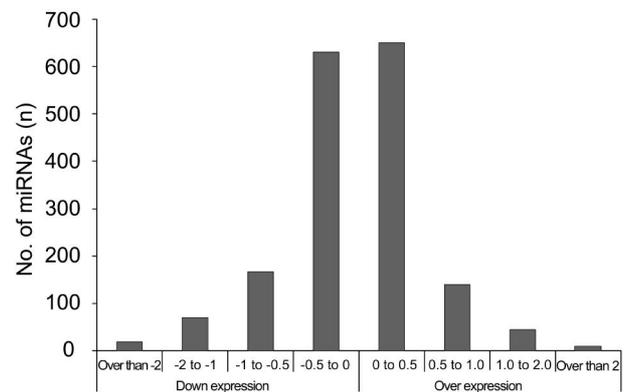
Figure 3 Screening of miRNAs Most miRNAs in pterygial fibroblasts were found from -1 to +1. X-axis indicates the log scale of miRNA expression in control and pterygial fibroblasts. Negative scale indicates downregulation, and positive scale indicates overexpression.

FITC-fluorescence (vimentin) were observed by confocal laser scanning fluorescent microscopy in both fibroblasts. Western blotting by using anti-vimentin antibody detected vimentin expression in both pterygial and control fibroblasts. Analysis of the nuclear morphology of fibroblasts with DAPI showed well-defined structures (Figure 2).

Primary miRNA Screening and Analysis Of the 1733 miRNAs screened, 887 were up-regulated and 846 were down-regulated in pterygial fibroblasts compared with those in control fibroblasts (Figure 3). In all, 90 (5.2%) miRNAs were down-regulated (log ratio of less than -1.0) and 55 (3.2%) miRNAs were up-regulated (log ratio of more than 1.0) in pterygial fibroblasts. Further, 1280 (73.9%) miRNAs showed a minimal change in expression compared with those in control fibroblasts. Of the 145 miRNAs that were differentially expressed in pterygial fibroblasts according to the log ratio test, 18 up-regulated miRNAs and 9 down-regulated miRNAs met the second criterion, *i.e.* base log value of >7.0. None of the down-regulated miRNAs met the repetitive test criterion. Further, 4/18 up-regulated miRNAs, namely, miRNA-143a-3p, miRNA-181a-2-3p, miRNA-377-5p and miRNA-411a-5p, showed consistent upregulation in repetitive tests. MiRNAs belonging to the miRNA-200
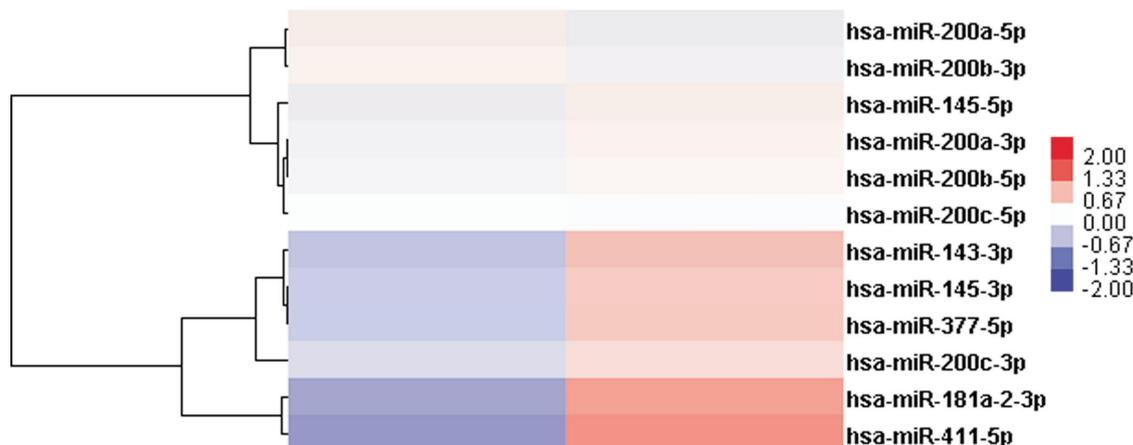
Figure 4 Heat map analysis Heat map of differentially expressed miRNA loci (left column: control fibroblasts; right column: pterygial fibroblasts).

family and miRNA-145-3p showed low expression in both pterygial and control fibroblasts; moreover, miRNA-145-5p showed minimal changes in expression (Table 1).

Secondary miRNA Analysis Primary screening of 1733 miRNAs identified 4 miRNAs (miRNA-143a-3p, miRNA-181a-2-3p, miRNA-377-5p and miRNA-411a-5p) that met all the above-mentioned criteria and were up-regulated in pterygial fibroblasts compared with control fibroblasts (Figure 4). Of the 4 target miRNAs, miRNA-143a-3p had the highest mean ratio compared with miRNAs in control fibroblasts (3.17±0.80). The mean ratios of miRNA-181a-2-3p, miRNA-377-5p, and miRNA-411a-5p were 1.72 ±0.22, 2.33±0.71, and 2.18±0.66, respectively (Figure 5).

DISCUSSION

Results of the present study showed that miRNA-143a-3p, miRNA-181a-2-3p, miRNA-377-5p and miRNA-411a-5p were up-regulated in pterygial fibroblasts compared with control fibroblasts, suggesting their involvement in the pathogenesis of pterygium.

miRNA-143a-3p had the highest overexpression rate among all the miRNAs analyzed. Few studies have been performed on miRNA-143a-3p, and it is suggested to be involved in the pathogenesis of cardiac morphogenesis and cancer [10-11]. miRNA-143 shows the lowest expression in various tumors and is thought to act as a tumor suppressor [12]. However, miRNA-143a-3p was overexpressed in pterygial fibroblasts in the present study, suggesting its involvement in the pathogenesis of pterygium. miRNA-181a-2-3p was also markedly up-regulated in pterygial fibroblasts compared with control fibroblasts. miRNAs belonging to the microRNA-181 family, including miRNA-181, are significantly up-regulated in human hepatocellular carcinoma cells [13]. miRNAs belonging to the miRNA-181 family play a critical role in PAH-induced hepatocarcinogenesis by targeting mitogen-activated protein kinase (MAPK) phosphatase-5, thus regulating p38 MAPK activation. Compared with healthy conjunctivas, pterygial tissues also contain activated

Table 1 Expression of the major miRNAs

miRNAs	Log values of miRNAs		Log ratio of miRNAs
	Pterygial fibroblasts	Control fibroblasts	
4 target miRNAs			
miRNA-143a-3p	13.61	12.32	1.28
miRNA-181a-2-3p	10.09	8.33	1.77
miRNA-377-5p	7.53	6.42	1.10
miRNA-411a-5p	9.65	7.68	1.96
Other miRNAs			
miRNA-145-3p	3.68	2.60	1.07
miRNA-145-5p	14.19	13.82	0.36
miRNA-200 family			
miRNA-200a-3p	1.63	1.43	0.20
miRNA-200a-5p	1.54	1.93	-0.39
miRNA-200b-3p	5.43	5.68	-0.25
miRNA-200b-5p	2.26	2.13	0.12
miRNA-200c-3p	5.44	4.76	0.68
miRNA-200c-5p	1.85	1.86	-0.003

Four target miRNAs showed upregulation and previous reported miRNA-145-3p and miRNA-145-5p were slightly upregulated. miRNA-200 family had diverse response.
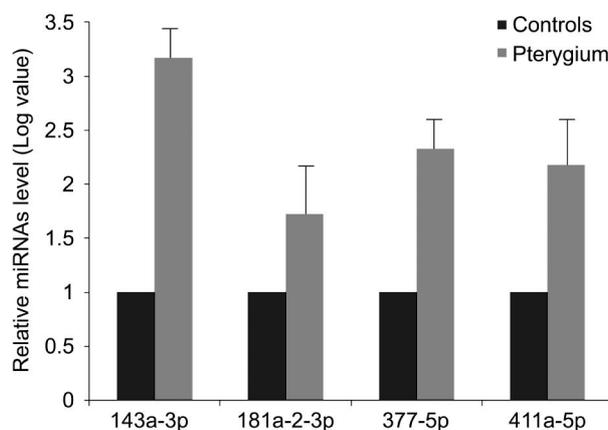
Figure 5 Analysis of the 4 target miRNAs The 4 miRNAs were significantly up-regulated in pterygial fibroblasts.

MAPKs [14]. Therefore, miRNA-181a-2-3p may play a key role in the pathogenesis of pterygium.

The results of the present study showed that miRNA-377-5p was up-regulated in pterygial fibroblasts compared with control fibroblasts. Wang *et al* [15] reported that overexpression of miRNA-377 in diabetic nephropathy indirectly increased the production of fibronectin. Pterygial tissues show increased expression of fibronectin and macrophage inflammatory protein-4 [16]. Therefore, the results of the present study suggest that miRNA-377-5p may be involved in increasing the levels of fibronectin in pterygium.

The results of the present study also showed that miRNA-411a-5p expression was up-regulated in pterygial fibroblasts compared with control fibroblasts. miRNA-411 belongs to the miRNA-379 family and is expressed from the miRNA-379/miRNA-656 cluster located within the *DLK-DIO3* region on human chromosome 14 [17]. Harafuji *et al* [18] reported that overexpression of miRNA-411 decreased the mRNA expression of YY1-associated factor 2 (*YAF2*), which negatively regulates muscle-restricted genes, in myoblasts. To date, no study has investigated *YAF2* expression in pterygial fibroblasts. However, microRNA-411a-5p may induce pterygium through other mechanisms.

Lan *et al* [19] reported that decreased miRNA-215 expression may increase fibroblast cycling and proliferation and induce pterygium formation. miRNA-221 may influence pterygium formation through *p27KIP1* [20]. In the present study, miRNA-215 and miRNA-211 were slightly down-regulated in pterygial fibroblasts (data not shown), suggesting that these 2 miRNAs were involved in the pathogenesis of pterygium.

Primary screening performed in the present study showed that miRNA-145-3p and miRNA-145-5p were up-regulated in pterygial fibroblasts. However, secondary analysis by performing PCR did not provide consistent results. This discrepancy might have resulted from the use of different methodologies in 2 studies. We analyzed pterygial fibroblasts in the present study and whole pterygial tissues in our previous study. In the present study, miRNAs belonging to the miRNA-200 family, including miRNA-200a-3p, miRNA-200a-5p, miRNA-200b-3p, miRNA-200b-5p, miRNA-200c-3p, and miRNA-200c-5p, did not show consistent expression, with miRNA-200a-3p, miRNA-200b-5p, and miRNA-200c-3p showing slight upregulation and miRNA-200a-5p, miRNA-200b-3p, and miRNA-200c-5p showing slight downregulation. However, overall expression of these miRNAs was low in both pterygial and control fibroblasts. The ratios suggesting differential regulation were not significant.

Thus, the results of the present study showed that 4 out of 1733 mature human miRNAs, namely, miRNA-143a-3p, miRNA-181a-2-3p, miRNA-377-5p, and miRNA-411a-5p were substantially up-regulated in pterygial fibroblasts compared with those in control fibroblasts. These results suggested that differential expression of these miRNAs is involved in the pathogenesis of pterygium. However, further studies should be performed to confirm these results.

ACKNOWLEDGEMENTS

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Retraction Notice

Retraction: "Brain derived neurotrophic factor keep pattern electroretinogram from dropping after superior colliculus lesion in mice" by Bin-Bin Yang, Xu Yang and Huai-Yu Ding published on *International Journal of Ophthalmology* 2016;9 (3): 369-372. DOI:10.18240/ijo.2016.03.07

This article has been retracted by the authors. This article involves in authorship dispute. After a thorough investigation, we regret to announce that this article must be retracted. We apologize to the readership of *International Journal of Ophthalmology.*

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The Press of International Journal of Ophthalmology (IJO Press)

1st July, 2016