Identification of pterygium-related mRNA expression profiling by microarray analysis

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Abstract

Purpose   Pterygium is a common degenerative and proliferative disease of the ocular surface. In some severe cases, pterygium may lead to irregular corneal astigmatism and corneal stromal scarring with visual impairment. The proliferative capacities of pterygial cells make them appear similar to tumorigenesis. Although considerable progress has been made towards understanding the etiology of the disease, the pathogenesis of pterygium has not been completely understood. In our previous study, we constructed pterygium-related lncRNA libraries by using microarray to investigate the potential roles of lncRNAs in pterygium. In this study, our objective was to explore the role of mRNA in pterygium.
Patients and methods   In this study, we constructed pterygium-related mRNA libraries by using microarray to investigate the potential roles of mRNAs in pterygium. Quantitative real-time PCR (qRT-PCR) was performed to validate some of the deregulated mRNAs in 10 patients.
Results   A total of 1485 upregulated and 2978 downregulated mRNAs were identified in pterygium tissues compared with paired adjacent normal conjunctival tissues (log fold change > 2.0). qRT-PCR was performed to validate four overregulated and two underregulated mRNAs in 10 patients.
Conclusions   Our results reveal differentially expressed mRNAs in pterygium and suggest that mRNAs may be the novel molecular targets for therapy of pterygium.
Eye (2017) **31,** 1733–1739; doi:10.1038/eye.2017.116; published online 30 June 2017

Introduction

Pterygium is a common ocular surface disease characterized by a triangular-shaped growth consisting of fibrotic subconjunctival connective tissue and hypertrophy of the overlying conjunctival epithelium.[1] Once the triangular-shaped growth covers the pupil, it becomes a significant sight-threatening complication. The biological mechanisms underlying pterygium are not well understood, and the only therapy employed when it affects vision is surgical removal.[2] But recurrences are common. Based on the pterygium's propensity for growth, there have been investigations suggesting that pterygium may be a proliferative disorder rather than a degenerative process.[1] Although considerable progress has been made toward understanding the etiology of the disease, the pathogenesis of pterygium has not been completely understood.[3,4] There is a significant need to gain more insights into pterygium formation and recurrence in order to enable the design of new therapeutic strategies, for inhibiting pterygium growth, regressing pterygia, or preventing recurrence.[5]

In our previous study, we constructed pterygium-related lncRNA libraries by using microarray to investigate the potential roles of lncRNAs in pterygium. Quantitative real-time PCR (qRT-PCR) was performed to validate three overregulated and two underregulated lncRNAs in 10 patients. We reported, for the first time, that lncRNAs were differentially expressed in pterygium compared with paired adjacent normal conjunctival tissues.[6]

In this study, we hypothesized that the targeted mRNAs of pterygium-related lncRNAs might also be involved in the molecular pathogenesis of pterygium. To test this hypothesis, the global expression profiles of mRNAs were analyzed by using microarray and qRT-PCR in human pterygium compared to normal conjunctiva. Our results are likely to provide important insights into the pathogenesis of pterygium.

Materials and methods

Patient samples preparation

This clinical study was approved by the ethical review committee of the Second Affiliated

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Hospital of Nanjing Medical University. The surgical specimens were handled in accordance with the Declaration of Helsinki. Written informed consent for specimen collection and subsequent analysis was obtained from every enrolled participant. Twelve patients referred for elective pterygium surgery provided written informed consent and were included in the study. All patients were in good health and ranged from 49 to 79 years of age, comprising five males and seven females. Clinical and demographic information for the patients was shown in our previous paper.[6] Conventional excision of pterygium with autotransplantation of a superotemporal conjunctival graft was carried out under local anesthesia. In the same patients, 1.5 × 1.5 mm of conjunctiva tissues were obtained from the inferior temporal quadrant of the bulbar conjunctiva during the surgery and served as control tissue. Clinical details pertaining to the study patients are described in Table 1 of our previous paper.[6] Whole pterygial tissues, including pterygial head and body, were submitted to the analyses. All the locations of pterygial tissues we collected were nasal. All tissues were immediately frozen in liquid nitrogen and stored in a − 80 °C freezer until use.

RNA extraction and quality control

To extract RNA from tissues, the frozen tissues were resuspended in TRIzol reagent (Life Technologies, Carlsbad, CA, USA) and finally eluted into 50 μl of elution solution according to the manufacturer's instructions. We measured RNA quantity and quality using NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE,

USA), and assessed RNA integrity using standard denaturing agarose gel electrophoresis. All the RNA samples were stored at − 80 °C until further processing. Complementary DNA (cDNA) was synthesized from 1 μg of total RNA by using a PrimesScript TM RT Master Mix Kit (TaKaRa, Dalian, China) with random hexamer primers in a final volume of 20 μl. The reverse transcription reactions were performed at 37 °C for 15 min, 85 °C for 5 s, and 4 °C for 10 min.

Microarray

Similar to our previous study,[6] lncRNA microarray analysis, including labeling, hybridization, scanning, normalization, and data analysis, was performed by Shanghai KangChen Biotech. Co., Ltd. (Shanghai, China).

RNA labeling and array hybridization

RNA labeling and array hybridization were carried out according to the Agilent One-Color Microarray-Based Gene Expression Analysis protocol (Agilent Technologies, Santa Clara, CA, USA) with minor modifications. After removal of rRNA, mRNA was purified from total RNA (mRNA-ONLY Eukaryotic mRNA Isolation Kit, Epicentre, Madison, WI, USA). Each sample was amplified and transcribed into fluorescent cRNA along the entire length of the transcripts without 3′ bias, utilizing a mixture of oligo(dT) and random primers (Arraystar Flash RNA Labeling Kit, Arraystar, Rockville, MD, USA). Then, the labeled cRNAs were purified using the RNeasy Mini Kit (Qiagen, Hilden, Germany). The

Table 1 Top 10 significantly differentially expressed mRNAs

Seqname	Gene symbol	Regulation	Fold change	P-value
ENST00000215531	C19orf77	up	69.7435792	0.03739714
NM_001005245	OR5M11	up	66.3547482	0.003698218
NM_002313	ABLIM1	up	63.7124451	0.005113739
NM_000451	SHOX	up	60.7777558	0.00645416
NM_004828	NCR2	up	54.5006866	0.03096669
NM_001003443	OR56A3	up	51.6560277	0.02631791
NM_001007272	DUSP13	up	50.1999544	0.002349316
NM_001178097	C12orf74	up	49.3215676	0.017149074
ENST00000333082	PSMF1	up	46.3302978	0.033824667
NM_002314	LIMK1	up	44.804016	0.014881755
NM_017912	HERC6	down	91.6195708	0.009542471
NM_018847	KLHL9	down	73.6666841	0.02165143
NM_001001676	LCN9	down	68.0751028	0.04796421
NM_016529	ATP8A2	down	67.2412715	0.049084569
NM_006019	TCIRG1	down	64.8387175	0.000474277
NM_004999	MYO6	down	63.0938027	0.023926299
ENST00000367324	PKP1	down	61.1732449	0.006679274
NM_020854	KIAA1468	down	57.3011683	0.018643498
NM_201429	RTN3	down	56.1974943	0.013278382
NM_005908	MANBA	down	55.0688815	0.005347933

concentration and specific activity of the labeled cRNAs (pmol Cy3 per μg cRNA) were measured using NanoDrop ND-1000 (NanoDrop Technologies) and 1 μg of each labeled cRNA was fragmented by adding 5 μl 10 × blocking agent and 1 μl of 25 × fragmentation buffer. Then, the mixture was heated at 60 °C for 30 min and 25 μl of 2 × GE hybridization buffer was added to dilute the labeled cRNA. Then, 50 μl of hybridization solution was dispensed into the gasket slide and assembled to the lncRNA expression microarray slide. The slides were incubated for 17 h at 65 °C in an Agilent Hybridization Oven. Finally, the hybridized arrays were washed, fixed, and scanned using the Agilent DNA Microarray Scanner (part number G2505C; Agilent Technologies).

Quantitative real-time reverse-transcription PCR

The selected mRNAs and the primers (Supplementary Information I) used for qRT-PCR were designed and synthesized by Generay Biotech (Shanghai, China). We used β-actin as an internal control for tissue samples. qRT-PCR analysis was carried out using ViiA 7 Real-time PCR System (Applied Biosystems, Foster City, CA, USA) with SYBR expression assay system (TaKaRa). The conditions for PCR reaction were: an initial denaturation at 95 °C for 10 min, followed by 40 PCR cycles at 95 °C for 10 s and 60 °C for 60 s, finally annealing and extension at 95 °C for 10 s, 60 °C for 60 s, and 95 °C for 15 s. Each sample was assayed in triplicates. The standard curve method was used to determine the fold change in gene expression in the pterygium samples relative to the adjacent normal conjunctival samples.

Gene ontology (GO) enrichment

The Gene Ontology (GO) project (http://www. geneontology.org) provides a controlled vocabulary to describe gene and gene product attributes.[7] GO categories were considered as significantly enriched only if the Fisher's exact probability had a P-value < 0.05.

Statistical analysis

We used Agilent Feature Extraction software (version 11.0.1.1) to analyze acquired array images. Quantile normalization and subsequent data processing were carried out by using the Gene Spring GX v12.1 software package (Agilent Technologies). mRNAs for which at least two out of four samples have flags in Present or Marginal ('All Targets Value') were chosen for further data analysis after quantile normalization of the raw data. Differentially expressed mRNAs with statistical significance between the two groups were identified through P-value or FDR filtering, and fold-change

filtering. Hierarchical clustering and combined analysis were performed by using homemade scripts.

All experiments were repeated at least three times. All data were expressed as the mean ± SEM. Statistical significance was analyzed by using SPSS 20.0 software package (SPSS Inc., Chicago, IL, USA). Differential expression levels of mRNAs were compared using independent-samples t-test between two groups. Fisher's exact test was used in GO analysis. $P < 0.05$ was considered statistically significant.

Results

Profile of microarray data

According to microarray expression profiling data, 21 360 mRNAs were detected. In addition, 4463mRNAs (log fold change > 2.0) were found differentially expressed between pterygium samples and its paired adjacent normal conjunctival tissue samples (Table 1, Supplementary Information II). All these mRNAs were obtained from authoritative databases, RefSeq, GENCODE, and many related literatures. Hierarchical cluster shows mRNA expression patterns (Figure 1a). Scatter plot was used for assessing the mRNA expression variation between the two groups of pterygium and adjacent tissues (Figure 1b). In all 1485 upregulated and 2978 downregulated mRNAs were identified in pterygium tissues compared with paired adjacent normal conjunctival tissues. A volcano plot filtering between two groups initially revealed differentially expressed mRNAs with statistical significance (fold change > 2.0, $P < 0.05$) (Figure 1c).

Real-time quantitative PCR confirmation

By using qRT-PCR, four upregulated mRNAs (DUSP13, ATP4A,LRRC20, and ST6GALNAC4) and two downregulated mRNAs (KIAA1468 and MANBA) with log fold changes > 10 were randomly selected to test and verify the microarray data in 10 different samples of pterygium tissues and paired adjacent normal conjunctival tissues (Figure 2). The qRT-PCR results and microarray data are consistent. Thus, microarray data profiling indicated a series of mRNAs constantly differentially expressed between pterygium tissues and paired adjacent normal conjunctival tissues.

Expression signatures of deregulated mRNAs between pterygium tissues and paired adjacent normal conjunctival tissues

As mRNA expression is tissue specific, to further study the mRNA expression pattern in pterygium tissues, we first investigated general signatures of deregulated

mRNAs with log fold changes >2.0, including mRNA length distribution and chromosome distribution. Classification analysis showed that most mRNAs in our microarray are distributed across 2001–3000 nt length (Supplementary Information III A). Chromosome distribution showed that up- and downregulated mRNAs have different chromosome locations (Supplementary Information III B).
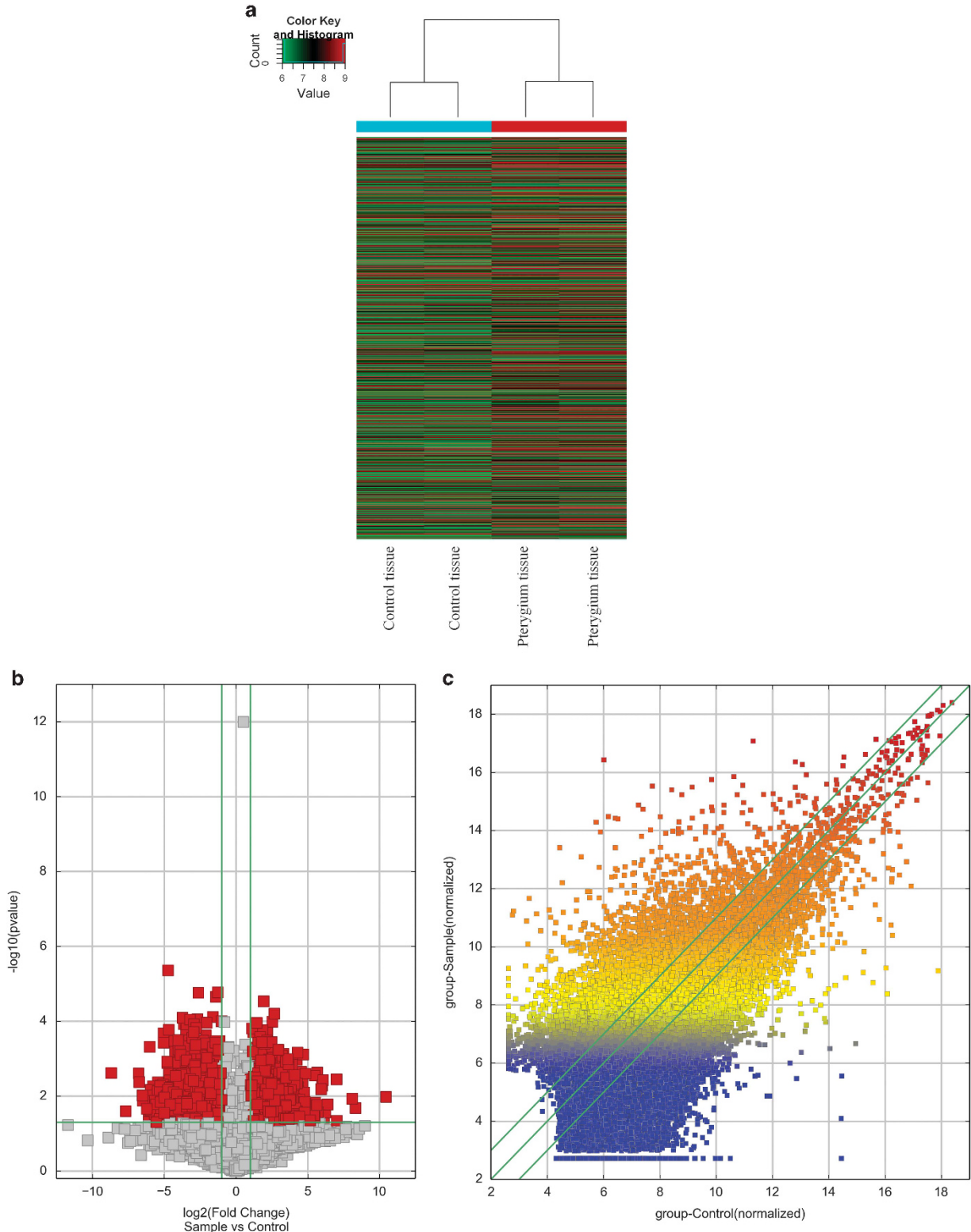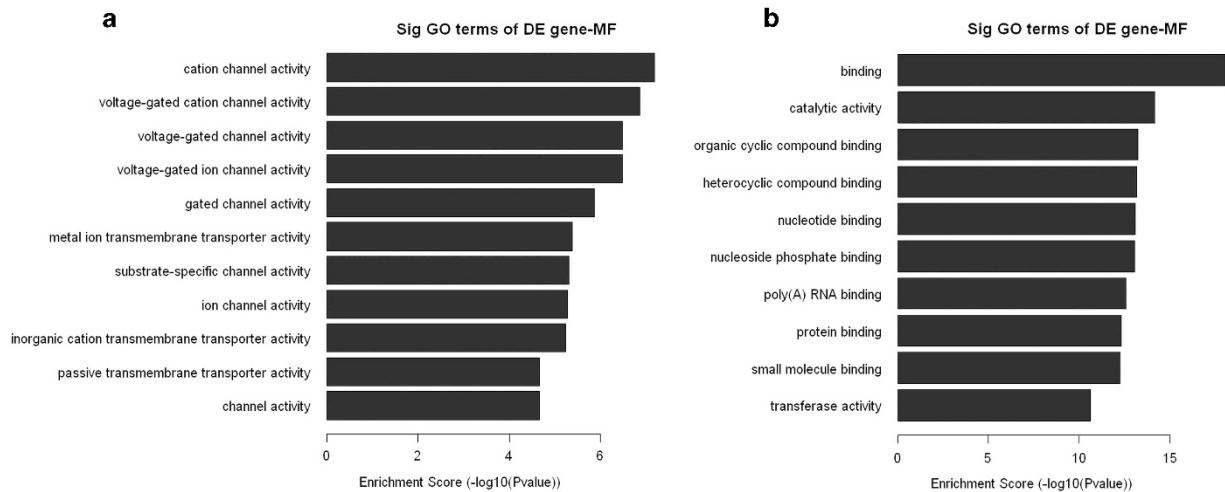
GO analysis

GO analysis was performed to determine the gene and gene product enrichment, which covered three domains: biological processes, cellular components, and molecular functions. Fisher's exact test was used to determine whether the overlap between the differentially expressed gene list and the GO annotation list was greater than that expected by chance (a *P*-value <0.05 is recommended). In our previous study, we found that the highest GO classification targeted by overregulated transcripts comprised the single-organism process (ontology: biological process), membrane (ontology: cellular component), and protein binding (ontology: molecular function). Meanwhile, the highest GO classification targeted by the underregulated transcripts consisted of the cellular process (ontology: biological process), cell part (ontology: cellular component), and binding (ontology: molecular function). To analyze the candidate molecular functions of mRNAs, we enriched the molecular functions of both up- and downregulated mRNAs. It is interesting that most molecular functions of upregulated mRNAs are involved in proliferation and differentiation, such as cation channel activity, voltage-gated cation channel activity, gated channel activity, and substrate-specific channel activity, which shows that upregulated mRNAs in pterygium tissues have a close association with proliferation and differentiation (Figure 3a). On the contrary, we found the molecular functions of downregulated mRNA to be enriched in binding, catalytic activity nucleotide binding, and protein binding (Figure 3b).
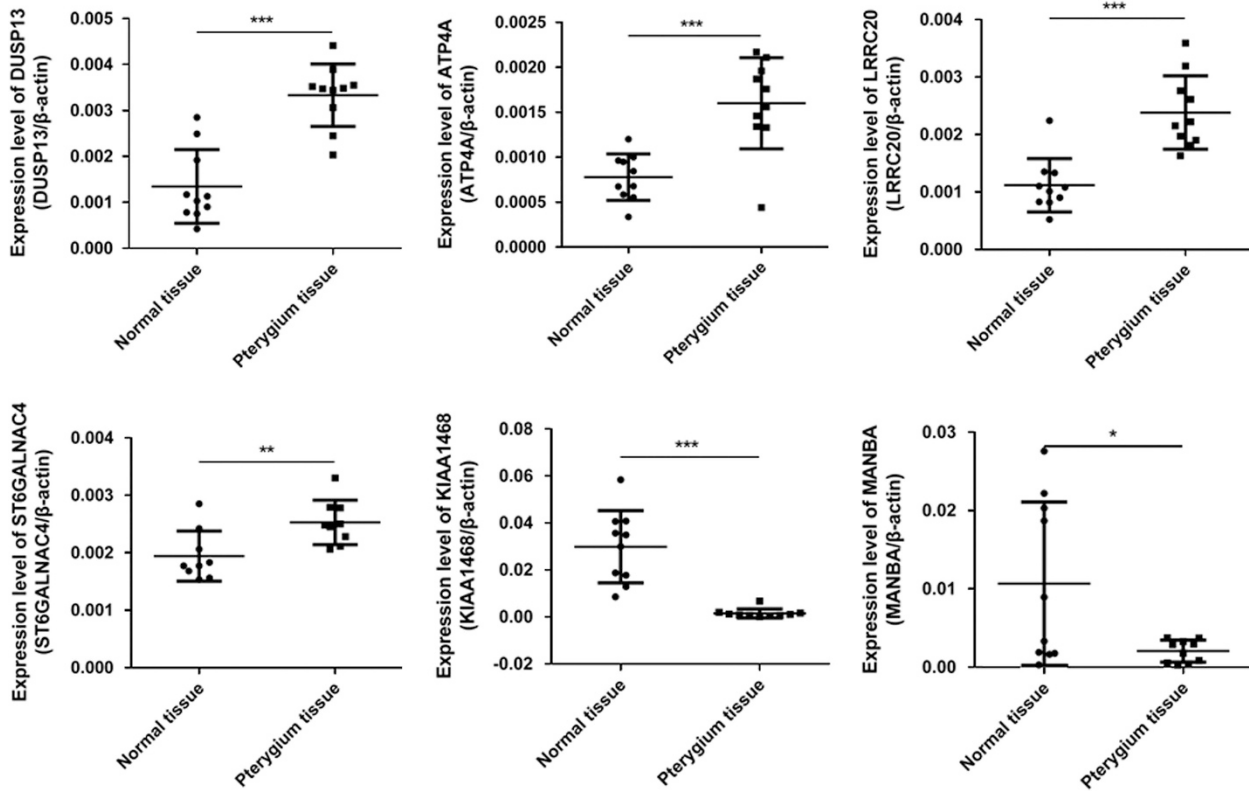
Figure 2 The differential expression of mRNAs between pterygium and paired adjacent normal conjunctival tissues were validated by qRT-PCR. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

Figure 1 The differentially expressed profiling in pterygium was compared with paired adjacent normal conjunctival tissues. (a) Differentially expressed mRNAs in pterygium and paired adjacent normal conjunctival tissues were analyzed using hierarchical clustering. Hierarchical clustering analysis arranges samples into groups by expression level, 'red' indicates high relative expression, and 'green' indicates low relative expression. (b) The scatter plot is a visualization method used for assessing the mRNAs expression variations between pterygium and paired adjacent normal conjunctival tissues. The values of the *X* and *Y* axes in the scatter plot are the averaged normalized signal values of the group (log2 scaled). The green lines are fold change lines. (c) Volcano plot analysis of mRNA microarray data of differentially expressed mRNAs between pterygium and paired adjacent normal conjunctival tissues. The red points in the plot represent the differentially expressed mRNAs with statistical significance (fold change > 2.0, $P<0.05$), while the vertical lines correspond to 2.0-fold up and down, and the horizontal line is $P = 0.05$.

a

Sig GO terms of DE gene-MF

b

Sig GO terms of DE gene-MF

Figure 3 GO analysis. The top 10 GO terms that associated with coding gene functions of upregulated mRNAs (a) and downegulated mRNAs (b) are listed.

Discussion

Pterygium is a progressively growing fibrovascular tissue mass originating outside the cornea, which often invades the optical axis. In some severe cases, pterygium may lead to irregular corneal astigmatism and corneal stromal scarring with visual impairment.[8] Environmental conditions may be involved in establishing the conditions on the ocular surface that allow the development of primary pterygium, ultraviolet light exposure, and other conditions that produce chronic irritation of the eye. Indeed, pterygium tissue shares the features of many pre-malignant tissues, including epithelial proliferation, goblet cell hyperplasia, angiogenesis, inflammation, and elastosis.[9,10] However, the mechanisms of pterygium formation have not been fully clarified. Figuring out the expression profile of mRNAs in pterygium is critical in understanding its pathogenesis.

Recent microarray analysis has demonstrated that microRNAs (miRNAs) and their targeted mRNAs are functionally important in processes relevant to pterygium pathogenesis.[11] The concerted upregulation of miR-221 was known to regulate downstream p27Kip1 gene expression,[8] and evidence showed that miR-200 family was a potential regulator of EMT in pterygium.[11] The proliferative capacities of pterygial cells have a mechanism similar to tumorigenesis. However, the roles of pterygium pathogenesis still remain unclear.

In the present study, we investigated the mRNA expression profiles of pterygium using microarray analysis and found that the mRNAs expression levels were altered compared to adjacent normal conjunctival tissues. From the microarray expression profiles, we found that 1485 upregulated and 2978 downregulated mRNAs were significantly differentially expressed

(>2.0-fold) in all pterygium samples. Six mRNAs were evaluated by qRT-PCR to validate the consistency. The qRT-PCR results and microarray data are consistent. The mRNA changes were observed both in pterygial stroma and epithelium, all of which expressed proliferative features compared to normal conjunctival tissues.

For pterygium, genetic mutations in developmental genes were common, as observed in other proliferative diseases such as cancers. An overregulated mRNA, ATP4A, was identified in a previous study to be closely correlated with the carcinogenesis of gastric cancer. It was considered a potential driver gene whose targets were seen to be involved in cell adhesion, angiogenesis, cell proliferation, and apoptosis.[12] The proliferative feature of ATP4A may help us learn more about the relationship between pterygium and mRNAs at the transcriptional level.

To analyze the candidate molecular functions of mRNAs, we enriched the molecular functions of both up- and downregulated mRNAs. The results indicate that most molecular functions of upregulated mRNAs, such as cation channel activity, voltage-gated cation channel activity, gated channel activity, and substrate-specific channel activity, are involved in proliferation and differentiation, which shows that upregulated mRNAs in pterygium tissues are closely related to proliferation and differentiation. This is the first report showing that pterygium-related mRNAs are associated with proliferation and differentiation by microarray. Meanwhile, we found the molecular functions of downregulated mRNA are enriched in binding, catalytic activity nucleotide binding, and protein binding.

In conclusion, we report for the first time that pterygium is associated with significant changes in expression of multiple mRNAs. Some of the changes

identified in this study suggest new mechanisms potentially relevant in the formation of pterygium. In the future, we will expand the number of enrolled patients and apply immunofluorescence, proteomics, or protein-level quantification of the mRNA targets in patients with pterygium staged according to severity level. Further investigation will help to evaluate the relevance of these mRNAs in the pathogenesis of pterygium, and their potential as biomarkers, molecular targets, and prognosis markers in the medical treatment of pterygium.

Summary

What was known before

- In our previous study, we constructed pterygium-related lncRNA libraries using microarray to investigate the potential roles of lncRNAs in pterygium. We reported for the first time that lncRNAs were differentially expressed in pterygium compared with paired adjacent normal conjunctival tissues.

What this study adds

- We report for the first time that pterygium is associated with significant changes in expression of multiple mRNAs. Our results reveal differentially expressed mRNAs in pterygium and suggest that mRNAs may be the novel molecular targets for therapy of pterygium.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on Eye website (http://www.nature.com/eye)