• Basic Research •

Identification of pathogenic genes of pterygium based on the Gene Expression Omnibus database

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Abstract

• AIM: To identify the pathogenic genes in pterygium.

• METHODS: We obtained mRNA expression profiles from the Gene Expression Omnibus database (GEO) to identify differentially expressed genes (DEGs) between pterygium tissues and normal conjunctiva tissues. The Gene Ontology, Kyoto Encyclopedia of Genes and Genomes pathway analysis, protein-protein interaction (PPI) network and transcription factors (TFs)-target gene regulatory network was performed to understand the function of DEGs. The expression of selected DEGs were validated by the quantitative real-time polymerase chain reaction (qRT-PCR).

• RESULTS: A total of 557 DEGs were identified between pterygium and normal individual. In PPI network, several genes were with high degrees such as *FN1, KPNB1, DDB1, NF2* and *BUB3. SSH1, PRSS23, LRP5L, MEOX1, RBM14, ABCA1, JOSD1, KRT6A* and *UPK1B* were the most downstream genes regulated by TFs. qRT-PCR results showed that *FN1, PRSS23, ABCA1, KRT6A, ECT2* and *SPARC* were significantly up-regulated in pterygium and *MEOX1* and *MMP3* were also up-regulated with no significance, which was consistent with the our integrated analysis.

• CONCLUSION: The deregulated genes might be involved in the pathology of pterygium and could be used as treatment targets for pterygium.

• KEYWORDS: pterygium; gene expression; protein-protein interaction network; pathogenesis

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INTRODUCTION

Pterygium is a general ocular surface and degenerative disease characterized by conjunctival fibrovascular proliferation and invasion of the peripheral cornea. Pterygium is composed of the head that invades the cornea, the neck that includes the superficial limbus and the body that overlie the sclera[1]. Diverse biological pathways including increase of mitotic proteins and protein implicated in tissue invasion were remarkably affected in pterygium. Additionally, fibrosis, inflammation, angiogenesis, collagen deposition and extracellular matrix breakdown are associated with pterygium[2]. Pterygium is noted to be more prevalent than 20% of some populations and typically affects the younger population and can potentially lead to blindness[3].

Different processes have been reported as part of the pathology of pterygium, including cytokines, growth factors, viral infections, genetic predisposition, anti-apoptotic mechanisms, immunological mechanisms and extracellular matrix remodeling[4]. Up to now, the major treatment for pterygium is surgical removal. However, it recurs aggressively after surgical excision, and further treatment modalities are wide excision, adjunctive radiotherapy and antimitotic chemotherapy[5]. Therefore, understanding the underlying molecular mechanism in pterygium is vital to design a strategy for the non-surgical therapy of pterygium and the prevention of disease recurrence after surgery[6].

Here, we obtained differentially expressed genes (DEGs) in pterygium. The functional annotation was performed to uncover the biological function of DEGs. The protein-protein interaction (PPI) network and transcription factors (TFs)-target gene regulatory network were constructed. The expression of candidate DEGs were validated by the quantitative real-time polymerase chain reaction (qRT-PCR). Our study was useful in identifying pathogenic genes in the diagnosis or therapy of pterygium.

SUBJECTS AND METHODS

Ethical Approval The study was conducted in accordance with the Declaration of Helsinki and was approved by the Research Ethics Committee of the First Affiliated Hospital of Bengbu Medical College. Informed written consent was obtained from all participants, and research protocols were approved by the ethical committee of our hospital.

Table 1 Three datasets in the GEO dataset

Dataset	Author	Platform	Samples (N:P)	Year
GES83627	Lan W[a]	GPL14550 Agilent-028004 SurePrint G3 Human GE 8×60K Microarray (Probe Name Version)	4:4	2016
GES51995	Lan W[b]	GPL14550 Agilent-028004 SurePrint G3 Human GE 8×60K Microarray (Probe Name Version)	4:4	2013
GES2513	Wong YW[7]	GPL96 [HG-U133A] Affymetrix Human Genome U133A Array	4:8	2006

GEO: Gene Expression Omnibus database. [a]https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE83627; [b]https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE51995.

Gene Expression Profiles Herein, we found datasets from the GEOdatabase (http://www.ncbi.nlm.nih.gov/geo/) with the keywords pterygium "[MeSH Terms] OR pterygium [All Fields]) AND "Homo sapiens"[porgn] AND "gse"[Filter]. The type of study was characterized as "expression profiling by array". Datasets were genome-wide expression data of pterygium tissues and/or normal conjunctiva tissues. The datasets should be normalized or original, and three sets of mRNA data of pterygium were selected. Three datasets were displayed in Table 1.

Identification of Differentially Expressed Genes The DEGs was calculated using Limma and metaMA packages and then p-values were adjusted for multiple comparisons by the Benjamini and Hochberg method. $P<0.05$ was considered as DEGs.

Functional Annotation of Differentially Expressed Genes The GSEABase package in R language was performed for GO (http://www.geneontology.org/) analyses and KEGG (http://www.genome. jp/kegg/pathway.html) pathway enrichment of DEGs. $P<0.01$ was considered as statistically significant.

Protein-Protein Interaction The database of BioGRID (http://thebiogrid.org) was performed to construct PPI network between proteins encoded by top 200 DEGs and other proteins. Then, a PPI network was built by the Cytoscape (http://cytoscape.org/).

Analysis of Potential Transcription Factors to Target Differentially Expressed Genes The corresponding promoters of the top 40 DEGs were obtained by UCSC (http://www.genome.ucsc.edu/cgi-bin/hgTables). The TFs involved in regulating these DEGs were derived from the match tools in TRANSFAC. The Cytoscape software was used to build TFs network.

Quantitative Real-time Polymerase Chain Reaction Here, eight pterygium patients and three normal individuals were included in our study from the Department of Ophthalmology, the First Affiliated Hospital of Bengbu Medical College on 2016. Both pterygium tissues and corresponding normal conjunctiva tissues were obtained and frozen in liquid nitrogen immediately for further experiment.

Total RNA was extracted using TRizol reagent (Invitrogen, CA, USA). RNA was reverse-transcribed using a SuperScript III Reverse Transcription Kit (Invitrogen, CA, USA). Quantitative real-time PCR were conducted using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) on Applied Biosystems 7300 (Applied Biosystems, Foster City, CA, USA). The human GAPDH were used as endogenous controls for gene expression in analysis. Relative expression of DEGs was analyzed using the $2^{-\Delta\Delta CT}$.

Table 2 Top 20 DEGs in pterygium

Gene ID	Gene symbol	*P*
Up-regulated genes		
54829	*ASPN*	1.64E-12
3853	*KRT6A*	9.04E-12
4016	*LOXL1*	2.42E-09
11081	*KERA*	9.25E-09
1474	*CST6*	1.16E-08
4222	*MEOX1*	1.33E-08
131578	*LRRC15*	1.77E-08
19	*ABCA1*	1.90E-08
7348	*UPK1B*	2.48E-08
11098	*PRSS23*	4.35E-08
Down-regulated genes		
3912	*LAMB1*	9.49E-05
1364	*CLDN4*	1.50E-04
3189	*HNRNPH3*	2.11E-04
54434	*SSH1*	2.83E-04
10432	*RBM14*	2.93E-04
55423	*SIRPG*	3.27E-04
9620	*CELSR1*	3.39E-04
8125	*ANP32A*	3.54E-04
91355	*LRP5L*	3.94E-04
9929	*JOSD1*	5.15E-04

DEGs: Differentially expressed genes.

RESULTS

Differentially Expressed Genes Analysis and Functional Analyses After chip data processing, a total of 557 genes (321 up-regulated and 236 down-regulated) were identified. Top 20 genes were listed in Table 2. And the heat map of all DEGs was demonstrated in Figure 1. To investigate the biological function of the identified DEGs in pterygium, all DEGs were performed to GO term and KEGG analyses. In top 15 GO term analyses, single-organism developmental process, movement of cell or subcellular component, localization of cell and cell motility were the significant enrichment in biological process (Figure 2). Receptor binding, protein complex binding, cell

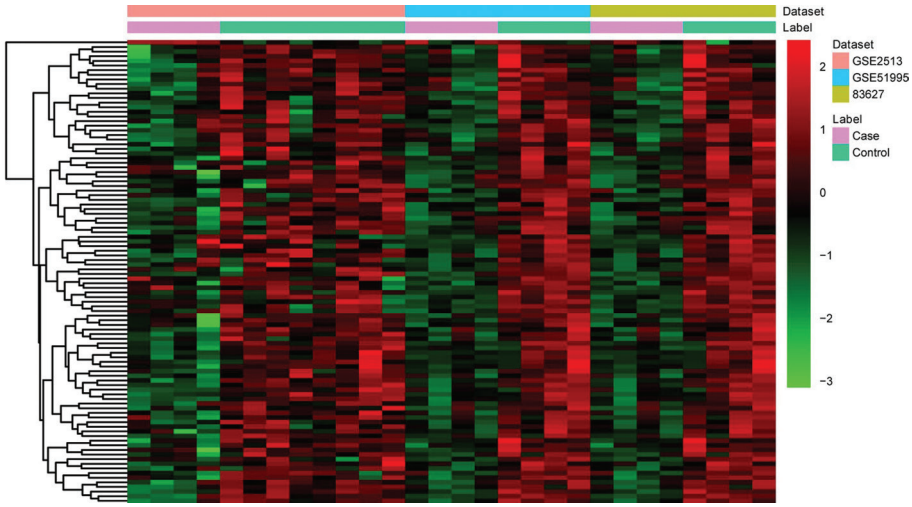

Figure 1 The heat map of top 100 DEGs Diagram presents the result of a two-way hierarchical clustering of top 100 DEGs and samples. Row and column represented DEGS and GEO data, respectively. The color scale represented the expression levels.

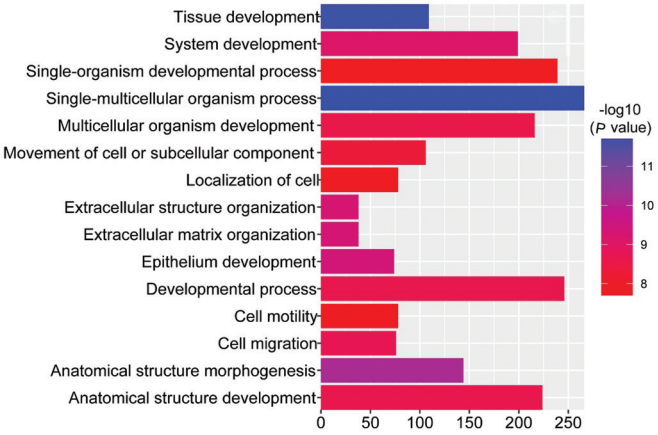

Figure 2 Top 15 significant enrichment biological processes of DEGs.

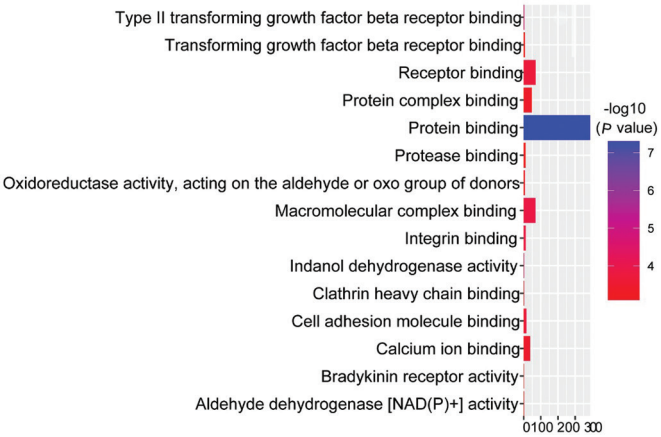

Figure 3 Top 20 significant enrichment molecular functions of DEGs.

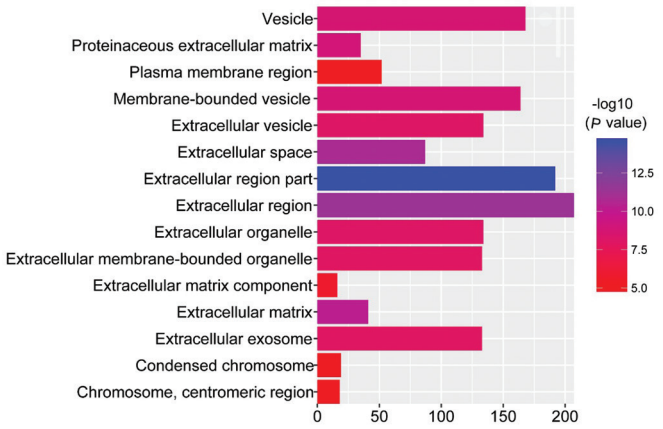

Figure 4 Top 20 significant enrichment cellular components of DEGs.

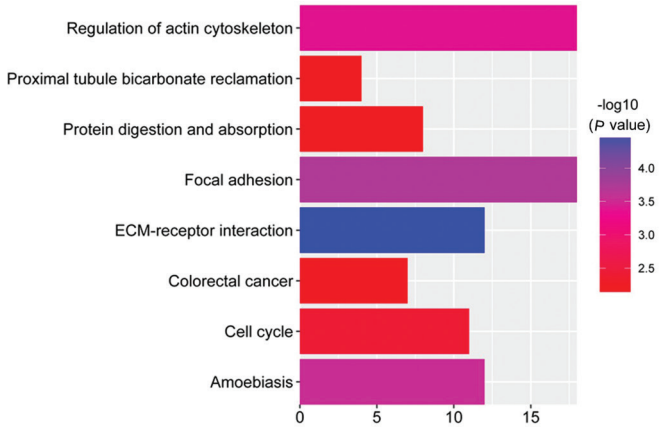

Figure 5 The KEGG enrichment analysis of DEGs.

adhesion molecule binding and calcium ion binding were the obviously enrichment in molecule function (Figure 3). Plasma membrane region, extracellular matrix component, condensed chromosome and chromosome, centromeric region were the most remarkably enrichment in cellular component (Figure 4). Additionally, proximal tubule bicarbonate reclamation, protein digestion and absorption were the significant enrichment signal pathway, which was shown in Table 3 and Figure 5, respectively.

Protein-Protein Intercation Network PPI network was built and visualized by Cytoscape, which was shown in Figure 6. The network consisted of 163 nodes and 191 edges. The proteins with high degrees were fibronectin 1 (*FN1*, degree=23), replication protein A2 (*RPA2*, degree=18), WNT signaling pathway regulator (*APC*, degree=15), estrogen receptor 1 (*ESR1*, degree=13), karyopherin subunit beta 1 (*KPNB1*, degree=8), damage specific DNA binding protein 1 (*DDB1*, degree=7), neurofibromin 2 (*NF2*, degree=7), mitotic

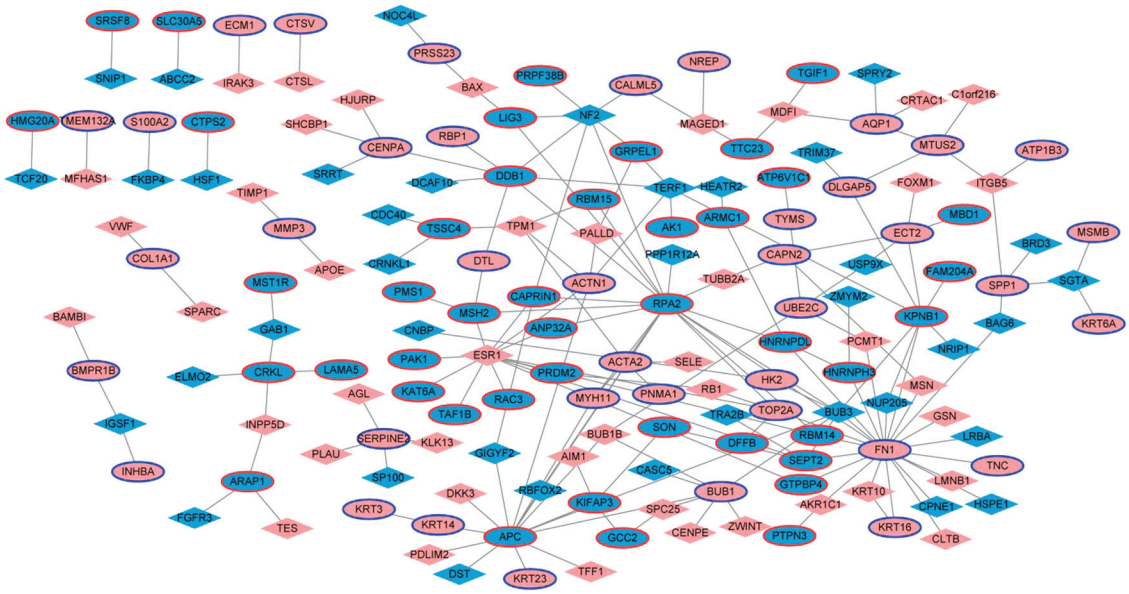

Figure 6 The PPI networks of top 200 DEGs.

Table 3 KEGG enrichment analysis

ID	Term	*P*	Count	Symbols
4512	ECM-receptor interaction	3.33E-05	12	*COL1A1;COL5A2;COL6A3;COMP;FN1;TNC;ITGB5;LAMA5;LAMB1;SPP1;VWF;TNN*
4510	Focal adhesion	1.92E-04	18	*ACTN1;CAPN2;COL1A1;COL5A2;COL6A3;COMP;CRKL;FN1;TNC;ITGB5;LAMA5;LAMB1;PPP1R12A;PAK1;RAC3;SPP1;VWF;TNN*
5146	Amoebiasis	2.92E-04	12	*ACTN1;ARG2;CD14;COL1A1;COL5A2;FN1;LAMA5;LAMB1;SERPINB13;SERPINB4;TGFB2; TGFB3*
4810	Regulation of actin cytoskeleton	4.18E-04	18	*ACTN1;APC;BDKRB1;BDKRB2;CD14;CRKL;F2R;FGFR3;FN1;GSN;ITGB5;MSN;PPP1R12A;PAK1;RAC3;GNA13;SSH1;MYH14*
4110	Cell cycle	3.85E-03	11	*BUB1;BUB1B;CCNA2;GADD45A;RB1;TGFB2;TGFB3;TTK;CDC45;BUB3;ORC6*
5210	Colorectal cancer	5.52E-03	7	*APC;BIRC5;BAX;MSH2;RAC3;TGFB2;TGFB3*
4974	Protein digestion and absorption	6.97E-03	8	*ATP1B3;COL1A1;COL5A2;COL6A3;PRSS2;SLC1A1;SLC15A1;SLC36A1*
4964	Proximal tubule bicarbonate reclamation	7.59E-03	4	*AQP1;ATP1B3;CA2;GLUD2*

KEGG: Kyoto Encyclopedia of Genes Genomes; ECM: The extracellular matrix.

checkpoint serine/threonine kinase (*BUB1*, degree=7) and mitotic checkpoint protein (*BUB3*, degree=7).

All the nodes are proteins encoded by DEGs and the blue and red borders ellipse represent proteins encoded by top 200 DEGs, respectively; The diamond represents the proteins encoded by other DEGs.

Transcription Factors Network We utilized TRANSFAC to obtain TFs regulating top 20 DEGs. In the end, we obtained TFs network consisting of 250 pairs of TFs-genes involving 36 TFs (Figure 7). The top 9 downstream genes covered by most TFs were slingshot protein phosphatase 1 (*SSH1*, degree=10), protease, serine 23 (*PRSS23*, degree=10), LDL receptor related protein 5 like (*LRP5L*, degree=8), mesenchyme homeobox 1 (*MEOX1*, degree=7), RNA binding motif protein 14 (*RBM14*, degree=7), ATP binding cassette subfamily A member 1 (*ABCA1*, degree=7), Josephin domain containing 1 (*JOSD1*, degree=6), keratin 6A (*KRT6A*, degree=6) and uroplakin 1B (*UPK1B*, degree=6). Six hub TFs were *Oct-1*, *Pax-4*, *Pax-6*, *Nkx2-5*, *AP-1* and *COMP1* (Table 4).

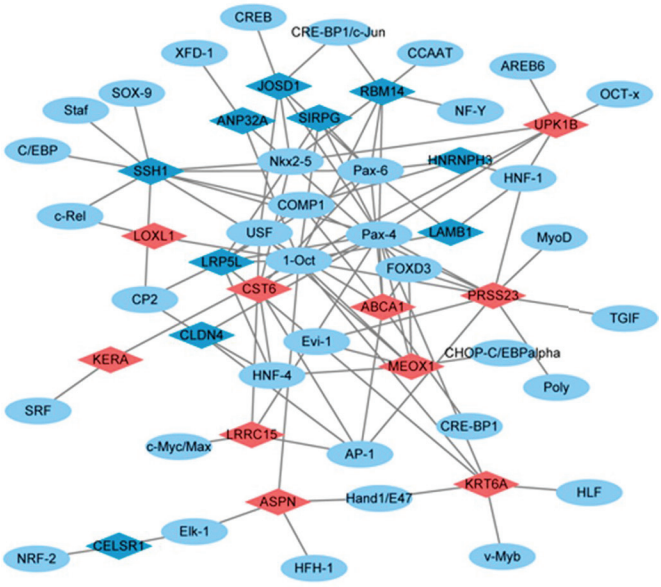

Figure 7 The TFs network The ellipse and diamond represent TFs and top 20 DEGs, respectively; the red and blue represent the up- and down-regulated DEGs, respectively.

Table 4 Top 6 TFs that covered the most target genes

TFs	Gene number	Target genes
1-Oct	13	*UPK1B, LAMB1, ASPN, RBM14, PRSS23, SSH1, KRT6A, KERA, LOXL1, ANP32A, MEOX1, SIRPG, LRP5L*
Pax-4	13	*LRRC15, UPK1B, CLDN4, ABCA1, RBM14, PRSS23, CST6, SSH1, KRT6A, MEOX1, SIRPG, LRP5L, JOSD1*
Pax-6	9	*LAMB1, ABCA1, HNRNPH3, RBM14, CST6, SSH1, SIRPG, LRP5L, JOSD1*
Nkx2-5	7	*UPK1B, RBM14, CST6, SSH1, SIRPG, JOSD1, LRP5L*
AP-1	5	*LRRC15, CLDN4, ABCA1, PRSS23, CST6*
COMP1	5	*ABCA1, HNRNPH3, PRSS23, SSH1, LRP5L*

TFs: Transcription factors.

Validation of Quantitative Real-time Polymerase Chain Reaction The expression of DEGs was quantified by qRT-PCR in blood samples of eight pterygium patients and three normal conjunctiva tissues. Eight DEGs including keratin 6A (*KRT6A*), ATP binding cassette subfamily A member 1 (*ABCA1*), mesenchyme homeobox 1 (*MEOX1*), protease, serine 23 (*PRSS23*), epithelial cell transforming 2 (*ECT2*), fibronectin 1 (*FN1*), matrix metallopeptidase (*MMP3*) and secreted protein acidic and cysteine rich (*SPARC*) were selected as candidate genes. As shown in Figure 8, *FN1* (P<0.05), *PRSS23* (P<0.01), *ABCA1* (P<0.05), *KRT6A* (P<0.05), *ECT2* (P<0.05) and *SPARC* (P<0.01), were significantly up-regulated in the pterygium compared to those of normal controls; The expression level of *MEOX1* and *MMP3* had no significance between pterygium and normal controls, but had the up-regulation tendency in pterygium. The expression pattern of all above DEGs was consistent with our integrated analysis.

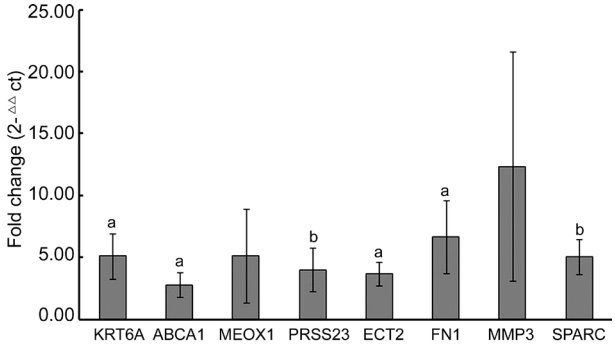

Figure 8 Validation DEGs in the pterygium by qRT-PCR
[a]P<0.05, [b]P<0.001 was considered as statistical significance.

DISCUSSION

It is significant to uncover the pathological mechanism of pterygium, which may provide a novel field in diagnosis and therapy of pterygium. In this study, we found several DEGs based on the GEO dataset, which was helpful in understanding the potential mechanism of pterygium and provide a new field in finding diagnosis biomarker and drug target of pterygium.

FN1 was found with the highest degree in the PPI network of pterygium. It is one component of the extracellular matrix and is found up-regulated in whole retina of extensive nerve injury[8]. In addition, *FN1* is increased in pterygia formation and recurrence[9]. Herein, we found up-regulated expression of *FN1* in pterygium. And qRT-PCR also validated the up-regulated tendency in pterygium. This suggested that the role of *FN1* in the process of pterygium and might constitute promising new target for pterygium.

KPNB1 is a differentially methylated gene in age-related macular degeneration[10]. In addition, the expression of *KPNB1* is up-regulated in optic nerve heads with elevated-intraocular pressure-induced injury of glaucoma[11]. *DDB1*, also called *XPE*, plays a crucial role in nucleotide excision global genome repair pathway. Defect of *DDB1* may be lead to pure xeroderma pigmentosum[12]. It is pointed out that patients with xeroderma pigmentosum are likely to develop ocular surface squamous neoplasia[12]. The inactivation of tumor-suppressor gene *NF2* is related to the formation of retinal and optic nerve lesions in neurofibromatosis type 2 patients[13], which is a clinically variable disease caused by *NF2* gene mutations. Additionally, it is reported that *NF2* is associated with dry eye disease[14]. *BUB3* is a human retinal endothelial protein identified by 2 or more peptides using multidimensional protein identification technology and plays a key role in the development of retinal[15]. Thus it can be seen that *PNB1*, *DDB1*, *NF2* and *BUB3* play a key role in the development of eye. Herein, we found that they were down-regulated in pterygium, which suggested the role in the process of pterygium. Further research is needed to validate their expression.

PRSS23 is found differentially expressed in ipsilateral retinal ganglion cells[16]. Furthermore, the expression of *PRSS23* is increased in the glaucomatous canine retina[17]. *MEOX1* is implicated in differentiation of mesoderm[18]. In mice, homozygous mutation of *MEOX1* gene has abnormalities in sclerotome and its derivatives[19]. Moreover, *MEOX1* plays an essential function in maintaining of sclerotome polarity and axial skeleton formation[19]. *ABCA1* is a cholesterol metabolic marker and mediates cholesterol efflux. The homozygous recessive mutation in the *ABCA1* gene may be result in the accumulation of lipid and cholesterol in cornea and reticuloendothelial system[20]. *ABCA1* is thought to play

essential roles in degenerative retinal diseases[21]. Chen W and Neale BM et al identified *ABCA1* as a risk factor for age-related macular degeneration[22]. In addition, it is significantly up-regulated in pterygium[23]. *KRT6A* gene encodes for proteins implicated in wound healing. In a genome wide expression data, the expression of *KRT6A* is remarkably increased by at least 2-fold in pterygium[23]. In addition, Tong *et al*[23] found that *KRT6A* is significantly up-regulated in pterygium compared to conjunctiva in microarray analysis. In this study, we found that *PRSS23*, *MEOX1*, *ABCA1* and *KRT6A* were all up-regulated in bioinformatics analysis and qRT-PCR validation. This suggested the association between *PRSS23*, *MEOX1*, *ABCA1*, *KRT6A* and pterygium and provided a new field for understanding the pathology mechanism of pterygium. It is reported that *SSH1* plays a role in Wnt5a-enhanced migration in human corneal endothelial cells[24]. It has been demonstrated that *RBM14* is down-regulated in primary visual cortex in dementia with lewy bodies[25]. *UPK1B* is an epithelial differentiation gene and functions in the cornea[26]. It is found up-regulated in the retinoblastoma tumors[27]. In this study, we found the down-regulated expression of *SSH1* and *RBM14* and up-regulated expression of *UPK1B* in pterygium. We speculated that *SSH1*, *RBM14* and *UPK1B* played pivotal roles in the visual function of pterygium.

ECT2 has been associated with tumor progression and is detected in a number of retinal progenitor cells[28]. Moreover, *ECT2* is expressed in response to chemotherapy induced stress in the retinoblastoma therapy[29]. Additionally, *ECT2* is over-expressed in the immortalized human corneal epithelial cell lines[30]. *MMPs* plays a crucial role in various eye diseases. And disordered expression of *MMPs* in the eye has been involved in different disorders, such as pterygium[31]. *MMP3*, one member of *MMPs*, is an attractive candidate for the pathogenesis and development of pterygium. Seet *et al*[1] and Di Girolamo *et al*[31] found that *MMP3* was up-regulated in pterygium. In addition, it is suggested that inhibitors of *MMP3* have a protective role against extracellular matrix degradation, which is a manifestation of pterygium. In our study, we found that *MMP3* was up-regulated in pterygium, which was in line with previous reports. This further indicated the role of *MMP3* in the pathology process of pterygium. *SPARC* is a matrix protein that plays roles in various biological processes including epithelial-mesenchymal transition, tissue fibrosis and angiogenesis in primary pterygium[9]. Additionally, *SPARC* is a well-known mediator in collagen production and deposition[32]. It is reported that the expression of *SPARC* is related to aberrant extracellular matrix deposition in pterygium[1]. In this study, *SPARC* was up-regulated in pterygium, which was consistent with previous reports. This suggested that *SPARC* may be complicated in the pathogenesis of pterygium.

In addition, there was no study reported that the association of other five DEGs (*RPA2*, *APC*, *ESR1*, *LRP5L* and *JOSD1*) with eye disease, especially pterygium, their potential roles in pterygium needs further research.

In summary, several DEGs including *FN1*, *KPNB1*, *DDB1*, *NF2*, *BUB3*, *PRSS23*, *MEOX1*, *ABCA1*, *KRT6A*, *SSH1*, *RBM14* and *UPK1B* play a pivotal role in the pathology of pterygium. Our findings may contribute to the identification of early diagnosis biomarker or drug target for pterygium.

There are limitations to this study. Firstly, the intensive molecular function of above genes wasn't investigated in this study. The *in vivo* and *in vitro* experiments are necessary for exploring the molecular function of these genes in the process of pterygium in the future work. Secondly, the sample size for qRT-PCR validation was small, additional studies with larger cohorts of pterygium are needed to validate and demonstrate the expression pattern of identified genes.

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Conflicts of Interest: Yue XL, None; **Gao ZQ,** None.

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