Elevation of S100 calcium-binding protein A7 in recurrent pterygium

YURU ZHANG[1] and FEI LIU[2]

[1]Department of Ophthalmology, Yantai Affiliated Hospital of Binzhou Medical University, Yantai, Shandong 264100; [2]Department of Ophthalmology, The Second Hospital of Dalian Medical University, Dalian, Liaoning 116027, P.R. China

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Abstract. Recurrent pterygium, a common ophthalmic disease, is difficult to treat as its pathogenesis is unclear. To investigate the key genes responsible for the recurrence of pterygium, tissue samples were collected from six patients with primary pterygium (primary group), six patients with recurrent pterygium (recurrent group) and six patients with ocular trauma (control group) who underwent surgery between December 2014 and June 2017. The differentially expressed genes amongst these tissues were detected using expression profiling microarrays and verified by reverse transcription-quantitative PCR (RT-qPCR). Comparing the primary and control groups, 10 genes, including PP7080, small proline-rich protein 2A, keratin 24, small proline-rich protein 2F, defensin β4A, serpin family A member 3, S100 calcium-binding protein A7 (S100A7), Fc fragment of IgG binding protein and BPI Fold Containing Family A Member 1, were identified to be consistently upregulated in recurrent pterygium tissues, whilst two genes (H19 imprinted maternally expressed transcript and secretoglobin family 2A member 1) were consistently downregulated. Following RT-qPCR verification, it was identified that that S100A7 gene was significantly upregulated in recurrent pterygium tissues compared with the other groups. Protein-protein interaction and Gene Ontology analysis further revealed that all genes interacting with S100A7 were mainly involved in the regulation of defense mechanisms against bacteria, mitogen-activated protein kinase (MAPK) pathway activation and receptor for advanced glycation end-products receptor binding. The present findings confirmed that elevation of S100A7 expression in recurrent pterygium may be associated with the inflammatory response and activation of the MAPK signaling pathway.

Correspondence to: Dr Fei Liu, Department of Ophthalmology, The Second Hospital of Dalian Medical University, 467 Zhongshan Road, Shahekou, Dalian, Liaoning 116027, P.R. China
E-mail: drliufei@dmu.edu.cn

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Introduction

Pterygium, a benign tumor of conjunctival tissues, affects visual function and ultimately leads to blindness (1). Recurrent pterygium usually occurs following primary pterygium excision, with recurrence rates reported to range from 1-31.2% (2). Pterygium is considered a degenerative condition (3) characterized by fibrovascular outgrowth of the conjunctiva over the cornea and is associated with cytokine and growth factor imbalance, immunological disturbances and genetic mutations (3). A previous study determined that human papillomavirus infection and genes associated with DNA repair, cell proliferation, migration and angiogenesis were critical in the development of pterygium (1).

The inadequate understanding of recurrent pterygium pathogenesis means that prevention is far from effective. Various surgical procedures and adjuvant treatments have been proposed to reduce post-operative pterygium recurrence, including radiation therapy, anti-metabolites, anti-angiogenetic factors and conjunctival auto-grafting, which are considered effective techniques to treat primary pterygium with low recurrence rates (2,4). Recurrence not only depends on a multitude of patient-related, clinical and surgical factors but is also closely associated with cellular molecular changes (4). The development of drug targets for pterygium treatment relies on a novel understanding of the specific appropriate molecular pathways; however, studies on the molecular mechanisms involved in recurrent pterygium are limited.

In the present study, expression profiling microarray analysis was performed to investigate the differentially expressed genes related to recurrent pterygium and their possible contributions to find a novel treatment target for recurrent pterygium.

Materials and methods

Sample collection. Conjunctival tissue samples were collected during surgery from 18 patients with pterygium and ocular trauma who underwent surgery between December 2014 and June 2017 in the Second Hospital of Dalian Medical University (Dalian, China) and Yantai Affiliated Hospital of Binzhou Medical University (Yantai, China). Patients included 10 males and 8 females between the ages of 34 to 67 years. The inclusion criteria consisted of patients diagnosed with primary

pterygium (n=6), recurrent pterygium (n=6) and ocular trauma (n=6). Those with other medical diseases were excluded. Patients were divided into the primary group, recurrence group and control group, respectively. Patients in the primary and recurrent groups were subjected to pterygium excision, whilst those in the control group received conjunctiva excision. Tissue samples were collected from the patients and stored in a nitrogen canister (-196˚C). The study was approved by the Institutional Ethical Committee of Yantai Affiliated Hospital of Binzhou Medical University and written informed consent was obtained from all patients.

Microarray analysis. Tissues from the primary, recurrent and control groups were homogenized and TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) was used to isolate RNA. Total RNA was quantified using NanoDrop 2000 (Thermo Fisher Scientific, Inc.). RNA samples (300 ng/sample) were used for the analysis of differentially expressed genes using the Gene Chip™ Human Genome U133 Plus 2.0 Array (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. When compared with the corresponding genes of two groups, those with absolute value of $\log_2$ fold change >1 and false discovery rate <0.05 were considered differentially expressed genes. The primary samples were assessed with the control and then the recurrent samples.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from primary pterygium, recurrent pterygium and normal conjunctival tissues with TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). A High Capacity cDNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used to synthesize cDNA with the following protocol: 94˚C for 3 min, then 94˚C for 30 sec; 58˚C for 30 sec and 72˚C for 5 sec for a total of 35 cycles. qPCR was performed with the Power SYBR Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: 94˚C for 3 min, 40 cycles of 94˚C for 20 sec and 60˚C for 60 sec, and then 72˚C for 10 min. All experiments were independently replicated three times with GAPDH as a control. Relative mRNA levels were quantified using the $2^{-\Delta\Delta Cq}$ method (5). Primer sequences were as follows: PP7080 forward, 5'-CCTGGTGATGTCCGACCTG-3' and reverse, 5'-CCATGAGCGCATCGCAATC-3'; small proline-rich protein 2A (SPRR2A) forward, 5'-AGTGCCAGCAGAAATATCCTCC-3' and reverse, 5'-TGCTCTTGGGTGGATACTTTGA-3'; small proline-rich protein 2F (SPRR2F) forward, 5'-AGTGCCAGCAGAAATATCCTCC-3' and reverse, 5'-GAACGAGGTGAGCCAAATATCC-3'; defensin β4A (DEFB4A) forward, 5'-CAGGATTGAAGGGACCTGTT-3' and reverse, 5'-CTTCACTTGGCCTGTGTGTC-3'; serpin family A member 3 (SERPINA3) forward, 5'-TGCCAGCGCACTCTTCATC-3' and reverse, 5'-TGTCGTTCAGGTTATAGTCCCTC-3'; S100 calcium-binding protein A7 (S100A7) forward, 5'-GATTGACAAGCCAAGCCTGC-3' and reverse, 5'-CAAAGACGTCGGCGAGGTAA-3'; Fc fragment of IgG binding protein (FCGBP) forward, 5'-CCTATGGAGCTGGTGGATACTC-3' and reverse, 5'-GCATAGTCAGAATGGATCACCA-3'; BPI Fold Containing Family A Member 1 (BPIFA1) forward, 5'-CTTGGCCTTGTGCAGAGC-3' and reverse, 5'-CAACAGACTTGCACCGACC-3'; H19 imprinted maternally expressed transcript (H19) forward, 5'-TACAACCACTGCACTACCTG-3' and reverse, 5'-TGGAATGCTTGAAGGCTGCT-3'; secretoglobin family 2A member 1 (SCGB2A1) forward, 5'-ACTCCTGGAGGACATGGTTGA-3' and reverse, 5'-TCTGAGCCAAACGCCTTGGGT-3'; keratin 24 (KRT24) forward, 5'-GCTATGATGGGGGCCTTCTC-3' and reverse, 5'-GCCACCTCCTCGTGGTTC-3' and GAPDH forward, 5'-CACTGCCAACGTGTCAGTGGTG-3' and reverse, 5'-GTAGCCCAGGATGCCCTTGAG-3'. All primers were designed by Primer Premier 5.0 (Premier Biosoft International) and synthesized by Sangon Biotech Co., Ltd.

Protein-protein interaction (PPI) and gene ontology (GO) analysis. To identify the key genes that interact with S100A7, a PPI network was constructed using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) online database (https://string-db.org/; version 10.5). All genes correlated with S100A7 were identified according to the strength of the data support (thickness of the line connecting the two genes) and a high-resolution bitmap was downloaded. For the interacting genes in the network, GO analysis was performed using functional enrichments function of the STRING database and biological processes and molecular functions were exported.

Statistical analysis. SPSS 20.0 software (IBM Corp.) was used for statistical analysis, and data are presented as the mean ± standard error. The independent Student's t test was used to compare relative mRNA levels in the microarray analysis. For multiple comparisons between groups, one-way analysis of variance was performed prior to running the Student-Newman-Keuls post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Identification of differentially expressed genes associated with recurrent pterygium. Compared with the control group, 78 genes were upregulated and 45 genes were downregulated in primary pterygium tissues determined using microarray analysis (P<0.05). The top 50 differential expressed genes are presented in Fig. 1A. There were 60 upregulated genes and 9 downregulated genes in recurrent pterygium tissues compared with primary pterygium tissues (P<0.05; Fig. 1B). To identify consistently differentially expressed genes associated with the recurrence of pterygium, the common differentially expressed genes between the three groups were selected (Table I). Compared with the control and primary groups, PP7080, SPRR2A, KRT24, SPRR2F, DEFB4A, SERPINA3, S100A7, FCGBP and BPIFA1 were consistently upregulated in recurrent pterygium whilst H19 and SCGB2A1 were consistently downregulated (Fig. 1C).

S100A7 is a marker associated with recurrence of pterygium. To further validate the aforementioned differentially expressed genes, RT-qPCR was performed on normal conjunctival, primary and recurrent pterygium tissues. Results revealed that S100A7 was upregulated in primary (P<0.05) and recurrent pterygium tissues (P<0.05); however, there were no significant

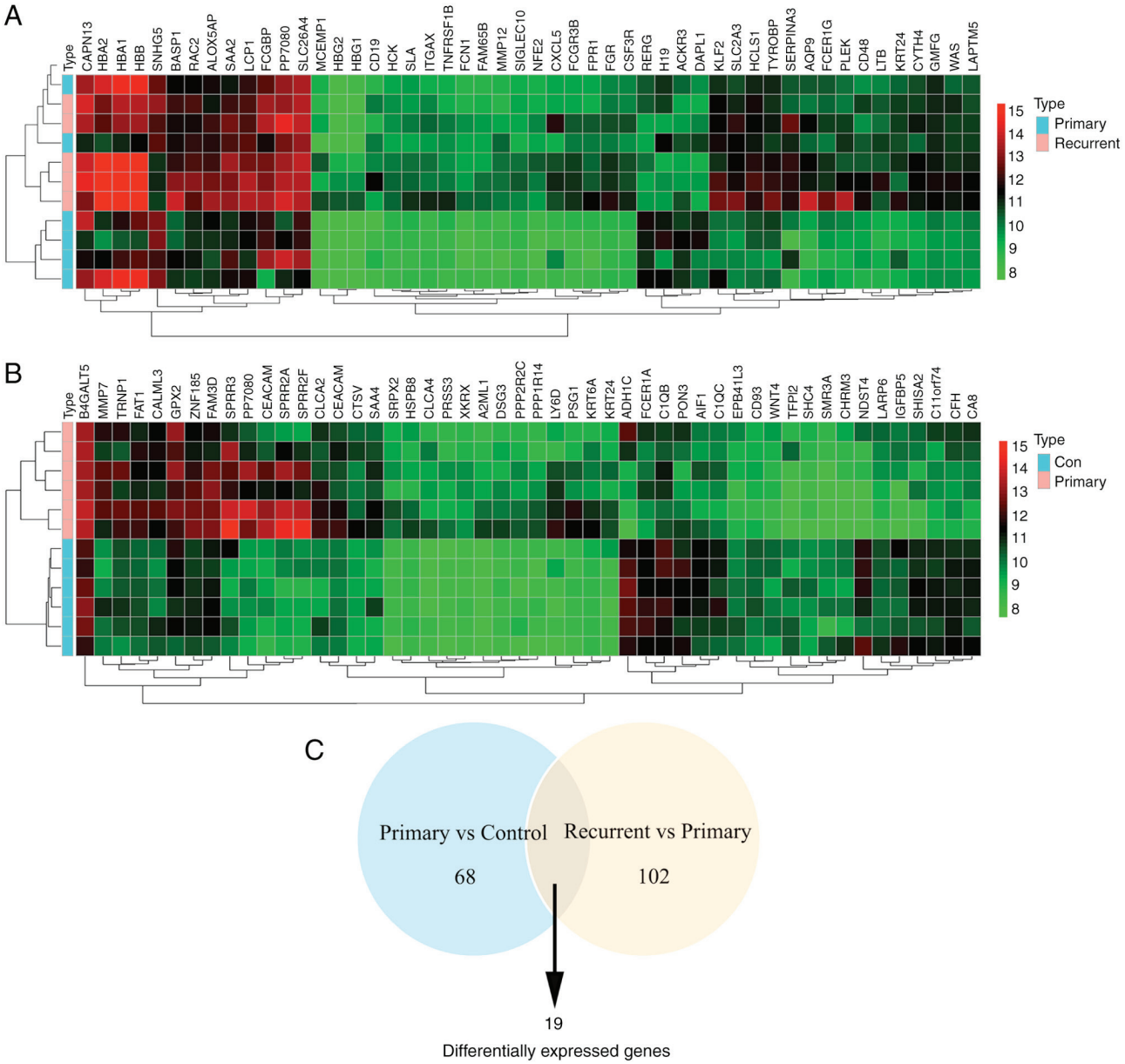


Figure 1. Differentially expressed genes in normal conjunctival tissues, primary and recurrent pterygium tissues. (A) Heatmap of differentially expressed genes between normal conjunctival tissues and primary pterygium tissues. (B) Heatmap of differentially expressed genes between primary and recurrent pterygium tissues. (C) The common differentially expressed genes amongst these three groups.

differences identified for any other genes (Fig. 2). These results suggested that S100A7 was closely associated with the recurrence of pterygium.

S100A7 regulates the immune defense mechanism and mitogen-activated protein kinase (MAPK) activation. As S100A7 was significantly upregulated in recurrent pterygium tissues, function enrichment was performed by PPI and GO analysis. PPI analysis suggested that S100A7 interacts with ten genes TATA-box binding protein, breast cancer type 1 susceptibility protein, MYC, protein max, S100A9, S100A12, ribonuclease 7, COP9 signalosome complex subunit 5, importin-5 and fatty acid-binding protein 5 (Fig. 3). Furthermore, GO analysis revealed that these genes were mainly involved in regulating defense mechanisms against bacteria, MAPK cascade activation (Table II) and receptor for advanced glycation end-products (RAGE) receptor binding (Table III).

Discussion

In the present study, an integrated analysis of differential gene expression was performed in primary and recurrent pterygium tissues by microarray analysis. It was identified then verified that S100A7 was significantly upregulated in recurrent pterygium tissues compared with primary pterygium and normal conjunctival tissues. Bioinformatics further demonstrated that S100A7 was associated with defense mechanisms against bacteria, MAPK cascade activation and RAGE receptor binding.

By reviewing worldwide academic research on pterygium recurrence following primary surgery, it was

Table I. Common differentially expressed genes in control group, primary and recurrent groups.

	Primary vs. Control			Recurrent vs. Primary		
Gene	Log_2 fold change	*t*	P-value	Log_2 fold change	*t*	P-value
PP7080	2.61	5.69	0.00	1.64	3.57	0.00
SPRR2A	2.87	4.65	0.00	1.35	2.19	0.04
KRT24	1.42	4.25	0.00	1.04	3.12	0.01
SPRR2F	2.71	4.23	0.00	1.36	2.13	0.05
TYROBP	-1.20	-3.76	0.00	1.11	3.48	0.00
DEFB4A	2.85	3.69	0.00	1.82	2.36	0.03
SERPINA3	1.68	3.53	0.00	1.91	4.03	0.00
S100A7	3.36	3.42	0.00	2.12	2.17	0.04
ALOX5AP	-1.07	-3.21	0.00	1.01	3.03	0.01
FCER1G	-1.02	-3.10	0.01	1.21	3.66	0.00
FCGBP	1.40	2.91	0.01	1.47	3.05	0.01
PLA2G2A	-1.27	-2.82	0.01	1.17	2.58	0.02
H19	-1.19	-2.79	0.01	-1.67	-2.46	0.00
BPIFA1	1.53	2.57	0.02	1.29	2.18	0.04
CAPN13	-1.08	-2.52	0.02	1.43	3.34	0.00
KRT12	1.34	2.47	0.02	-1.29	-2.37	0.03
SCGB2A1	-1.04	-2.46	0.02	-1.02	-2.41	0.03
CYP1B1	-1.72	-2.29	0.03	1.91	2.54	0.02
FPR1	-1.02	-2.17	0.04	1.47	3.12	0.01

Control, ocular trauma group; Primary, primary pterygium group; Recurrent, recurrent pterygium. *t*, t value from a Student's t test.

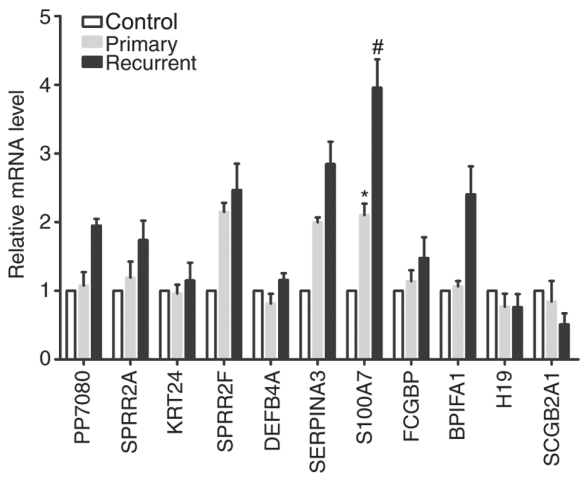


Figure 2. Differentially expressed genes were verified by reverse transcription-quantitative PCR analysis. *P<0.05 vs. Control and #P<0.05 vs. Primary. Control, ocular trauma group; Primary, primary pterygium group; Recurrent, recurrent pterygium; SPRR2A, small proline-rich protein 2A; KRT24, keratin 24; SPRR2F, small proline-rich protein 2F; DEFB4A, defensin β 4A; SERPINA3, serpin family A member 3; S100A7, S100 calcium-binding protein A7; FCGBP, Fc fragment of IgG binding protein; BPIFA1, BPI Fold Containing Family A Member 1; H19, H19 imprinted maternally expressed transcript; SCGB2A1, secretoglobin family 2A member 1.

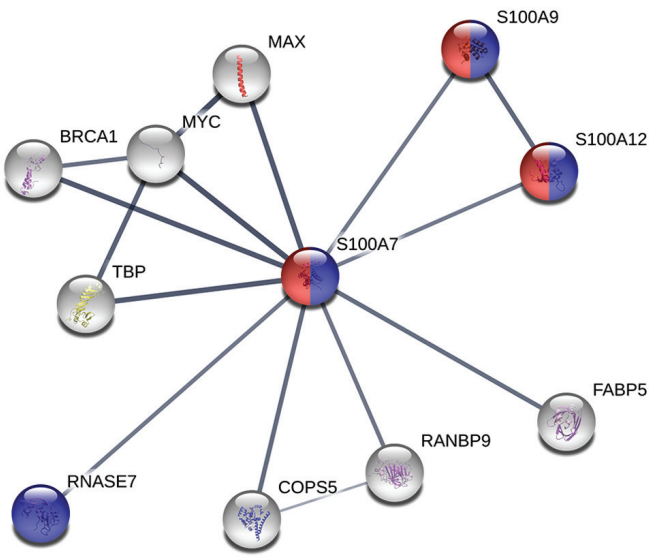


Figure 3. Protein-protein interaction network of all gene interactions with S100A7. BRCA1, breast cancer type 1 susceptibility protein; MAX, protein max; TBP, TATA-box binding protein; S100, S100 calcium-binding protein A7; RNASE7, ribonuclease 7; COPS5, COP9 signalosome complex subunit 5; RANBP5, importin-5; FABP5, fatty acid-binding protein 5.

determined that age, sunlight exposure, limbal stem cell deficiency and a large pterygium extent were relevant to pterygium recurrence (6). At the molecular level, vascular endothelial growth factor (VEGF) mRNA was observed in recurrent pterygium tissues (7) with abnormal expression of heat shock protein 90 also associated with pterygium recurrence (8). However, studies investigating the molecular mechanisms of pterygium remain limited. In the present

Table II. Biological processes of genes that interact with S100A7.

Pathway ID	Pathway description	FDR	Proteins in network
GO.0042742	Defense response to bacterium	0.028	RNASE7, S100A12, S100A7, S100A9
GO.0043408	Regulation of MAPK cascade	0.0423	COPS5, MYC, RANBP9, S100A12, S100A7
GO.0045893	Positive regulation of transcription, DNA-template	0.0423	BRCA1, COPS5, MYC, S100A12, S100A9, TBP
GO.0051238	Sequestering of metal ion	0.0423	S100A7, S100A9
GO.1902531	Regulation of intracellular signal transduction	0.0423	COPS5, MYC, RANBP9, S100A12, S100A7, S100A9
GO.0009966	Regulation of signal transduction	0.0464	BRCA1, COPS5, MYC, RANBP9, S100A12, S100A7, S100A9

GO, Gene Ontology; FDR, false discovery rate.

Table III. Molecular function (GO) of genes that interact with S100A7.

Pathway ID	Pathway description	FDR	Proteins in network
GO.0050786	RAGE receptor binding	$3.99x10^{-5}$	S100A12, S100A7, S100A9

GO, Gene Ontology; FDR, false discovery rate; RAGE, receptor for advanced glycation end-products.

study, it was demonstrated that the level of S100A7 was obviously increased in patients with recurrent pterygium, which suggested that S100A7 may be a potential target for the treatment of recurrent pterygium.

The S100A7 protein is overexpressed in hyper proliferative skin diseases, exhibits anti-microbial activities against bacteria and induces immunomodulatory activities (9). In the present study, elevation of S100A7 was observed in patients with recurrent pterygium; however, it could not be determined with certainty whether the upregulation of S100A7 was a cause or consequence (or both) of recurrent pterygium. According to previous studies, S100A7 is mainly associated with the occurrence and development of tumors and tissue hyperplasia (10,11). Additionally, in psoriasis patients, S100A7 was also determined to be overexpressed in the epidermis of psoriatic skin lesions (11). In addition, S100A7 is closely associated with autoimmune diseases (12), chronic inflammatory and angiogenesis (12,13). Pterygium is considered a chronic inflammatory disease caused by external stimulation and is manifested as degeneration, hypertrophy, hyperplasia and angiogenesis of conjunctival and sub-conjunctival tissues (14). Hence, the present study hypothesized that S100A7-mediated inflammation may be the main cause of pterygium recurrence. To investigate the potential underlying mechanism, PPI and GO analyses were performed, and the results suggested that all genes interacting with S100A7 were also involved in the immune defense against bacteria and the MAPK inflammatory response pathway. Furthermore, molecular function analysis identified that RAGE receptor binding is a major molecular function of S100A7. RAGE, a nucleic acid receptor, can promote the inflammatory response to DNA (15). S100A7 reportedly upregulates the expression of VEGF and promotes endothelial cell proliferation, which also involves RAGE receptor binding (16). Therefore, the aforementioned results revealed that S100A7-mediated inflammation may be one of the main causes of the recurrence of pterygium. In fact, S100 proteins, such as S100A8 and S100A9, are all regulatory proteins that mediate cell growth, apoptosis, differentiation and inflammation (17); however, abnormal expression of other S100 family members was not investigated in this study.

For S100A7 to be established as a biomarker of pterygium recurrence and a potential therapeutic target, advanced research utilizing larger datasets is required. The present study had certain limitations and can be expanded in several ways. First, it will be interesting to compare the expression of S100A7 with S100A6, S100A8 and S100A10 then analyze their correlation with other differentially expressed genes. Second, different S100A proteins should be investigated to determine whether they might be superior markers and if the expression of these genes is regulated by S100A7. Third, investigation into whether S100A7 is correlated with the size and thickness of primary and recurrent pterygium tissues should be performed. Finally, the link between the recurrence of pterygium and the inflammatory response or MAPK activation needs to be investigated in future studies.

The present study determined that elevation of S100A7 expression was observed in patients with recurrent pterygium, therefore, the gene may have potential as a therapeutic target for treatment of pterygium recurrence.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YZ performed the experiments, analysis and interpretation of data. FL designed the study and wrote the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Yantai Affiliated Hospital of Binzhou Medical University. Written informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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