Regulation of Apoptosis by miR-122 in Pterygium via Targeting Bcl-w

Yu-Hong Cui,[1] Hong-Yang Li,[2,3] Zi-Xun Gao,[4] Na Liang,[3] Sheng-Sheng Ma,[3] Fan-Jian Meng,[3] Zhi-Jie Li,[4] and Hong-Wei Pan[4,5]

[1]Department of Histology and Embryology, Guangzhou Medical University, Guangzhou, China
[2]Department of Ophthalmology, Guangdong No.2 Provincial People's Hospital, Guangzhou, China
[3]Department of Ophthalmology, Guangzhou Red Cross Hospital, the fourth affiliated hospital of Jinan University, Guangzhou, China
[4]Institute of Ophthalmology, School of Medicine, Jinan University, Guangzhou, China
[5]Department of Public Health and Preventive Medicine, Jinan University, Guangzhou, China

Correspondence: Hong-Wei Pan, Institute of Ophthalmology, School of Medicine, Jinan University, 601 West Huangpu Avenue, Guangzhou 510632, China; panhongwei@hotmail.com.

Y-HC and H-YL contributed equally to the work presented here and should therefore be regarded as equivalent authors.

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Purpose. To identify the differently expressed micro (mi) RNAs in pterygium compared with normal conjunctiva and investigate the potential role of miRNAs in the pathogenesis of pterygium.

Methods. With microRNA microarray and quantitative RT-PCR, we identified that microRNA-122 (miR-122) was significantly decreased in pterygium tissue. We detected the expression of Bcl-w, a predicted target of miR-122, in both pterygium and normal conjunctiva, as well as its correlation with the expression of miR-122. Pterygium epithelial cells were isolated and cultured, and transfected with miR-122 mimic or miR-122 inhibitor to change the miR-122 levels. The regulation of Bcl-w expression by miR-122 was examined with luciferase activity assay, quantitative (q) RT-PCR, and Western blot. The effect of the miR-122 on the apoptosis of cultured pterygium epithelial cells was investigated with TUNEL staining and caspase activity assay.

Results. We found the expression of Bcl-w, with an inverse correlation with the expression of miR-122, was significantly increased in pterygium, especially in the superficial layer of epithelium. In cultured pterygium epithelial cells, miR-122 could specifically combine with Bcl-w mRNA, and negatively regulated the expression of Bcl-w. Suppression of miR-122 could reduce apoptosis and caspase activity in pterygium epithelial cell treated with TNFα/cycloheximide (CHX), and this effect was abolished by inhibition of the expression of Bcl-w with specific siRNA.

Conclusions. Decreased expression of miR-122 in pterygium might result in abnormal cell apoptosis via its regulation of the expression of Bcl-w, and subsequently contribute to the development of pterygium.

Keywords: pterygium, apoptosis, miR-122, Bcl-w

Pterygium is a common disease characterized by abnormal fibrovascular proliferation and invasion on the ocular surface. It is generally accepted that ultraviolet radiation exposure is an important risk factor for pterygium. The lesion is gradual ingrowth of conjunctival tissue invading the cornea, typically triangular in shape, causing irritation, astigmatism, or visual axis occlusion. The pathogenesis of pterygium is a complicated process involving inflammation, neovascularization, extracellular matrix remodeling, and abnormal proliferation and apoptosis, and so on. However, the mechanism of pterygium development and formation is still not well understood.

Previous studies demonstrated that the epithelial cells played an important role in the development of pterygium because these cells underwent epithelial-mesenchymal transition (EMT) and regulated the secretion of matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs), which might be responsible for the invasive and recurrent behavior of this lesion.[1–3] Abnormal apoptosis was found in pterygium compared with normal conjunctiva. Also several studies showed that the expression of many apoptosis-related genes or proteins were different between pterygium and normal conjunctiva, indicating the role of apoptosis in pathogenesis of pterygium.[4–6] It is thought that pterygium might arise as a result of incorrect control of cellular apoptosis rather than from an increase in proliferative capacity.[7] Therefore, the regulation of pterygium epithelial cell apoptosis seems to play a key role in the pathogenesis of pterygium.

MicroRNAs (miRNAs), a group of endogenous and small (20–25 nt) noncoding RNAs, have a major role in posttranscriptional repression of protein expression through binding to the 3′ untranslated region (UTR) of mRNA transcripts to induce mRNA degradation or translational inhibition of target mRNAs.[8] A base-pairing complement between the seed sequence of miRNAs and the 3′UTR of their target mRNAs is the key determinant of specific recognition. It is estimated that 1000 human miRNAs are expressed by mammalian genome, and they are thought to regulate a large number of gene transcripts. Therefore, miRNAs have broad implications in regulation of a large variety of cellular processes including cell growth,

proliferation, differentiation, death, and apoptosis.[9–13] We speculate that miRNAs might be involved in the abnormal apoptosis in pterygium tissues.

In this study, we identified miR-122 was downregulated in pterygium compared with normal conjunctiva with an inverse correlation with the expression of Bcl-w, and miR-122 regulated pterygium epithelial cells apoptosis via targeting Bcl-w. These results demonstrate that miR-122 plays a role in pathogenesis of pterygium and thus might be a potential therapeutic or drug target for pterygium.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM)/F12 medium, fetal bovine serum (FBS), 0.25% trypsin-EDTA, TRIzol reagent, and Lipofectamine 2000 reagent were purchased from Invitrogen-Gibco (Carlsbad, CA, USA); 6-, 24-, and 96-well culture plates as well as cell culture flasks were from Corning (Corning, NY, USA). Caspase-Glo 3/7 assay kit, DeadEnd Fluorometric TUNEL System and Dual-Luciferase Reporter Assay System were purchased from Promega (Madison, WI, USA). MicroRNA-122 (miR-122) mimic, inhibitor and negative controls were from RiboBio Co., Ltd (Guangzhou, China). Bcl-w antibody, Bcl-w specific siRNA, control siRNA, horseradish peroxidase-conjugated secondary antibody, and FITC-labeled secondary antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase-conjugated glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was from KangChen Bio-tech Inc. (Shanghai, China). SABC immunohistochemistry kit was from Beijing ZhongShan Biotechnology Co. Ltd. (Beijing, China). Halt Protease Inhibitor Cocktail were from Pierce (Rockford, IL, USA). The Quick Start protein assay kit was purchased from Bio-Rad (Hercules, CA, USA). The enhanced chemoluminescence kit was purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). All reagent grade chemicals were from Sigma-Aldrich Corp. (St. Louis, MO, USA) unless otherwise indicated.

Patients and Specimens

Specimens of pterygium were obtained from 20 patients with primary pterygium who underwent surgical resection of the lesion (mean age 65.9 ± 10.2 years). Specimens of normal conjunctival tissue were obtained from 20 age-matched patients who underwent cataract surgery (mean age 69.1 ± 9.1 years). All patients were treated at the Department of Ophthalmology, the fourth affiliated hospital of Jinan University, Guangzhou, China. This study was approved by the institutional ethics review board at the fourth affiliated hospital of Jinan University and complied with the tenets of the Declaration of Helsinki for Research Involving Human Tissue, with written informed consent obtained from each patient. After surgical resection, the clinical specimens were properly treated or preserved for microarray, quantitative (q) RT-PCR, immunohistochemistry, or primary cell culture.

RNA Extraction

Total RNA was isolated using TRIzol (Invitrogen-Gibco) and miRNeasy mini kit (QIAGEN, Hilden, Germany) according to manufacturer's instructions, which efficiently recovered all RNA species, including miRNAs. RNA quality and quantity was measured by using nanodrop spectrophotometer (ND-1000; Nanodrop Technologies, Wilmington, DE, USA) and RNA integrity was determined by gel electrophoresis.

MicroRNA Microarray Analysis

MicroRNA expression profiles of pterygium tissues from pterygium patients and normal conjunctival tissues from patients undergoing cataract surgery were generated by applying the miRCURY Locked Nucleic Acid (LNA) microarray platform (Exiqon, Vedbaek, Denmark). All procedures were carried out according to the manufacturer's protocol. Briefly, 1 μg total RNA was dual-labeled with dyes spectrally equivalent to the Cy3 and Cy5 fluorophores, using a miRCURY Array Power Labelling kit (Exiqon). Labelled miRNAs were used for hybridization on a miRCURY LNA microRNA Array (v.18.0) according to array manual. Following hybridization, the slides were achieved, washed several times using Wash buffer kit (Exiqon), and finally dried by centrifugation for 5 minutes at 20*g*. Then the slides were scanned using the Axon GenePix 4000B microarray scanner (Axon Instruments, Foster City, CA, USA). Scanned images were then imported into GenePix Pro 6.0 software (Axon Instruments) for grid alignment and data extraction. The patients in pterygium group and the patients in cataract group, were pooled to represent the study and the control group, respectively. Differentially expressed miRNAs were defined as genes whose expression in the study group was consistently altered 2-fold (either greater or less) compared with the control group.

Real-Time PCR Analysis

For quantitative analysis of miRNA, total RNA was polyadenylated and reverse transcribed using a One Step PrimeScript miRNA cDNA Synthesis Kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. For quantitative analysis of Bcl-w mRNA, total RNA was reverse-transcribed using a PrimeScript RT reagent Kit (TaKaRa). Quantitative PCR was performed in an Applied Biosystems StepOne real-time PCR system using a SYBR Premix Ex Taq II Kit (TaKaRa). The forward primer for miR-122 was 5′-tggagtgtgacaatggtgtttg-3′, and the reverse primer for miRNAs was the Uni-miR qPCR Primer offered by the One Step PrimeScript miRNA cDNA Synthesis Kit. The Bcl-w specific primer was 5′-tttggttcggctttatcagg-3′(forward) and 5′-gaggactgc gagttccaaag-3′ (reverse). All reactions were performed in triplicate. U6 small nuclear RNA and GAPDH were used as internal controls for miRNAs and mRNAs, respectively. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method.

Immunohistochemistry

All specimens were fixed in 10% neutral buffered formalin and embedded in paraffin. Three-micrometer sections were cut, mounted on glass, and dried overnight at 37°C. All sections were then deparaffinized in xylene, rehydrated with alcohol, and washed in PBS. This buffer was used for all subsequent washes. Immunohistochemical study was conducted using the streptavidin-biotinperoxidase method, which was performed on paraffin-embedded tissues with anti–Bcl-w antibody. The primary antibody replaced by IgG was used us a negative control.

Isolation and Culture of Pterygium Epithelial Cells

Pterygium epithelial cells were isolated and cultured by using the explants methods reported previously with modification.[14,15] Briefly, the head of a fresh pterygium specimen was cut into small pieces (1–2 mm in diameter) under a stereomicroscope, washed in Hanks solution, and placed in a culture dish as explants in DMEM/F12 medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin. Epithelial cells migrated from explants as early

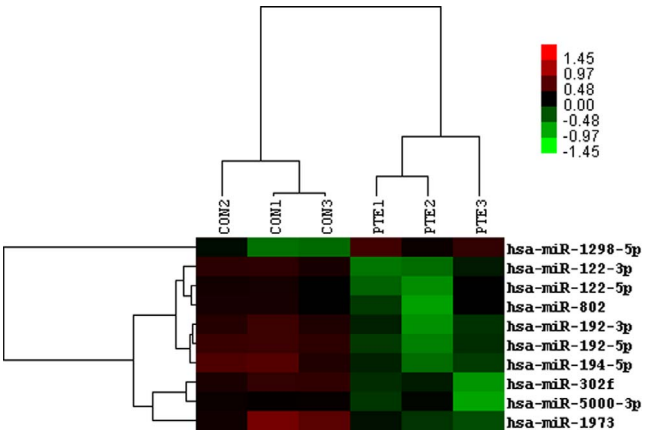

Figure 1. Hierarchical clustering of the miRNAs with a significantly different expression between pterygium and normal conjunctiva ($P < 0.05$). *Rows* represent individual genes; columns represent individual tissue samples. *Red* and *green* color indicate high and low expression, respectively. MicroRNA clustering tree is shown at *right*. Branch lengths of the dendrogram at *top* of the panel represent the similarity of the expression pattern between the samples. The scale represents the intensity of gene expression.

as 3 days in culture and displayed typical cobblestone morphology. The culture medium was replaced three times a week after the appearance of an outgrowth of cells from the explants. Fibroblast contamination was minimized by removing the tissue when a sufficient number of epithelial cells surrounded each explant. Primary pterygium epithelial cells were passaged for subcultures with 0.25% trypsin/0.02% EDTA solution. Cells in the third to sixth passages were used for the experiments described in the present study.

Cell Transfection With RNA Oligonucleotides

Pterygium epithelial cells were transiently transfected with miR-122 mimic (50 nM), miR-122 mimic negative control (50 nM), miR-122 inhibitor (100 nM), or miR-122 inhibitor negative control (100 nM) using Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's protocol. At 48 hours after transfection, treated cells were used for TUNEL analysis or harvested for luciferase activity assay, qRT-PCR, and Western blot.

Plasmid Construction and Dual Luciferase Assay

Human Bcl-w 3′-UTR (wild type, WT) and its identical sequence with a mutation in the miR-122 seed sequence (mutant, Mut) were amplified by PCR, inserted into the dual-luciferase reporter vector pmiR-RB-REPORT (Ribobio Co., Guangzhou, China) between the restrictive sites *Xho*I and *Not*I, to generate recombinant plasmids pmiR-RB-3′UTR-WT and pmiR-RB-3′UTR-Mut. These plasmids contained a synthetic Renilla luciferase gene (hRluc) encoding Renilla luciferase as the reporter and a synthetic firefly luciferase gene (hluc) encoding firefly luciferase as the internal control. The recombinant plasmids were confirmed by DNA sequencing and enzyme digestion. Transient transfections were performed using Lipofectamine reagent according to the manufacturer's recommendations (Invitrogen). Forty-eight hours post transfection, firefly and Renilla luciferase activities were consecutively measured using the Dual-Luciferase Assay System (Promega). The Renilla luciferase signal was normalized to the firefly luciferase signal for each individual analysis.

Western Blot

Western blot was carried out as described previously.[16] Briefly, 50 μg of protein from cell lysates was mixed with sample buffer, separated on 10% SDS-PAGE, transferred to nitrocellulose membranes (Pall Corporation, East Hills, NY, USA), and then blocked overnight in 5% defatted milk powder in Tris-buffered saline-0.1% Tween 20 (TBS-T). The membranes were probed with anti–Bcl-w primary antibodies, and subsequently washed with TBS-T and incubated with the secondary antibodies conjugated with horseradish peroxidase for 1 hour at room temperature. Glyceraldehyde 3-phosphate dehydrogenase immunoblotting was performed as an internal control for equal loading. After extensive washing in TBS-T, the blots were developed with enhanced chemiluminescence reagents.

Analysis of Cell Apoptosis

Cells grown on glass coverslips in 24-well plates were transfected with miR-122 inhibitor with or without Bcl-w specific siRNA or negative control siRNA. At 48 hours after transfection, pterygium epithelial cells were treated with TNFα (20 ng/mL) plus cycloheximide (CHX) (25 μg/mL) for 4 hours, and then fixed with 4% paraformaldehyde followed by permeabilization with 0.1% Triton X-100. After this, the protocol was followed as recommended by the manufacturer (DeadEnd Fluorometric TUNEL System; Promega). 4′,6-diamidino-2-phenylindole (DAPI) counterstain was used to visualize nuclei. TUNEL-positive cells were scored in at least five fields for each coverslip, and at least 1000 cells were counted for each coverslip.

Caspase 3/7 Activity Assay

Caspase 3/7 activity was determined as described previously.[16] Briefly, cells cultured in 96-well white-walled plates were transfected with miR-122 inhibitor with or without Bcl-w specific siRNA or negative control siRNA. At 48 hours after transfection, pterygium epithelial cells were treated with TNFα (20ng/mL) plus CHX (25μg/mL) for 4 hours. Caspase 3/7 activity was measured by Caspase-Glo 3/7 assay kit (Promega) according to the manufacturer's instructions. The luminescence of each sample was measured in triplicate with a microplate-reading luminometer (Safire II; Tecan, Crailsheim, Germany).

Statistical Analysis

Results were expressed as means ± SD. The differences between groups were analyzed using Student's *t*-test or, when multiple comparisons were made, 1-way ANOVA with post hoc Tukey's test. The relationship between the miR-122 and Bcl-w mRNA expression was analyzed using Pearson's correlation analysis. Statistical analyses were performed with SPSS (version 13.0; SPPS, Chicago, IL, USA). Values of P less than 0.05 were considered statistically significant.

Results

Differential Expression of miRNAs in Pterygium Compared With Normal Conjunctiva

The miRNA microarray analysis was performed in three pairs of pterygium tissues from primary pterygium patients and normal conjunctival tissue from age-matched cataract patients. Only those miRNAs altered by at least 2-fold with statistical difference between the two groups ($P < 0.05$) were considered to be significant candidates. The results of microarray demonstrated that 10 miRNAs were differently expressed between the

Table. miRNA Expression Profile of Pterygium Tissues Versus Normal Conjunctival Tissues

miRNA	Fold Change	*P* Value
hsa-miR-1298-5p	2.571	0.019
hsa-miR-122-3p	0.068	0.005
hsa-miR-122-5p	0.135	0.037
hsa-miR-192-3p	0.299	0.006
hsa-miR-192-5p	0.204	0.0002
hsa-miR-194-5p	0.175	0.015
hsa-miR-302f	0.354	0.006
hsa-miR-802	0.244	0.039
hsa-miR-1973	0.416	0.036
hsa-miR-5000-3p	0.406	0.047

The threshold value used to screen up- and downregulated miRNA was more than 2-fold or less than 0.5-fold change.

pterygium and normal conjunctiva specimen (Fig. 1). Only one miRNA, miR-1298-5p, showed increased expression in pterygium compared with normal conjunctiva. Nine miRNAs were downregulated in pterygium compared with normal conjunctiva, with miR-122-3p exhibiting the greatest decrease (Table). We found that the miR-122-5p and miR-122-3p, which were two mature microRNAs originated from opposite arms of the same pre-miRNA and were denoted with a −3p or −5p suffix, were both remarkably reduced in the pterygium. MicroRNA-122-5p, which is also termed as miR-122, has been proved to have important biological functions. Therefore, we selected miR-122 as an object to investigate its role in pterygium.

Bcl-w Expression was Increased in Pterygium Tissues and Inversely Correlated With miR-122

We used an online tool for miRNAs target prediction, TargetScan (in the public domain, http://www.targetscan.org/) to identify the downstream targets of miR-122. From several candidates, we selected an apoptosis-related protein, Bcl-w, as a putative miR-122 target in pterygium. A previous study reported the posttranscriptional regulation of Bcl-w expression by miR-122 in hepatocarcinoma.[17] However, both the expression and the action of microRNAs are tissue-specific, and so far there is no report about the role miR-122 in pterygium. So we first investigated Bcl-w expression in pterygium and its correlation with miR-122. We detected Bcl-w expression in pterygium and normal conjunctiva with immunohistochemistry and qRT-PCR. Histologic examination showed that pterygium tissues had thickened epithelium with underlying blood vessels and cell-enriched connective tissues in comparison with normal conjunctiva tissue (Figs. 2A, 2B). Bcl-w immunoreactivity was mainly found in the basal layer of epithelium in the normal conjunctival tissues (Fig. 2C). However, immunohistochemistry for Bcl-w proteins demonstrated that Bcl-w was abundantly expressed in the whole epithelium as well as in the blood vessels endothelium in the connective tissues of the pterygium tissues (Fig. 2D).

To verify the results of the microRNAs microarray analysis, we performed qRT-PCR in another 10 pairs of clinical specimen (10 normal conjunctiva and 10 pterygia). The results of qRT-PCR showed the average expression level of miR-122 in pterygium was less than one-third of the normal conjunctiva ($P < 0.05$), which was similar to the results detected by microarray, indicating the consistence of the results by these two miRNAs detection methods (Fig. 3A).

To compare the difference in the expression of Bcl-w mRNA between pterygium and normal conjunctiva, we analyzed

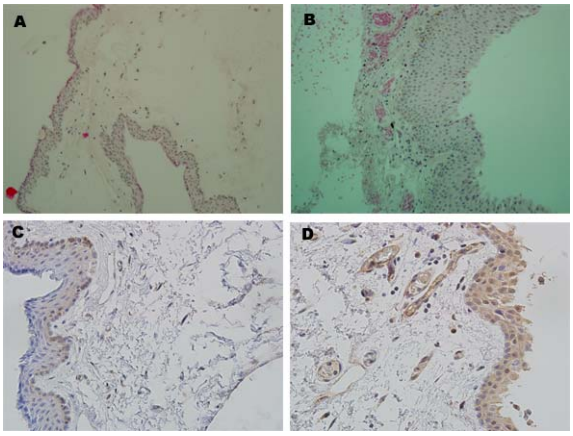

Figure 2. The morphologic characteristics and Bcl-w protein expression in pterygium and normal conjunctiva. Morphologic characteristics of conjunctiva and pterygium was observed with hematoxylin and eosin staining, at a magnification of 100. The normal conjunctiva (**A**) had well-defined basement membrane between the epithelium and underlying connective tissue, containing fewer blood vessels, and cells in the connective tissues. In contrast, the pterygium tissues (**B**) had thickened epithelium and contained enriched blood vessels and more cells in the connective tissues. With immunohistochemistry, Bcl-w expression was detected in paraffin sections of pterygium and normal conjunctiva, at a magnification of 200. Immunoreactivity for Bcl-w protein was found mainly in the basal layer of the normal conjunctival epithelium (**C**), but barely in the intermediate and superficial layer. In contrast, Bcl-w was expressed in the whole epithelium of pterygium (**D**).

RNAs extracted from 10 human pterygium tissues and 10 age-matched human normal conjunctival tissues by qRT-PCR. The results showed that the pterygium tissues exhibited significantly higher expression of Bcl-w mRNA, which had 2.26-fold increase on average compared with that in normal conjunctiva (Fig. 3B). Using Pearson's correlation analysis, we found a statistically significant inverse correlation between the miR-122 and Bcl-w expression (Fig. 3C, $r = -0.683$, $P < 0.05$). Taken together, these results indicated that miR-122 expression was inversely correlated with Bcl-w expression in human pterygium and normal conjunctiva.

Bcl-w was a Direct Target of miR-122 in Pterygium Epithelial Cells

By bioinformatics analysis, the 3′UTR of Bcl-w mRNA contained a complementary site for the seed region of miR-122 (Fig. 4A). We performed experiments to validate whether Bcl-w was a direct target of miR-122 in pterygium epithelial cells. A human Bcl-w 3′UTR fragment containing WT or mutant miR-122 binding sequence was cloned in a dual luciferase reporter gene pmiR-RB-Vector (Fig. 4B). In pterygium epithelial cells transfected with the reporter plasmids containing WT 3′UTR of Bcl-w, the luciferase activity was significantly suppressed by miR-122 mimic to 38.7 ± 3.2% of the negative control but increased by miR-122 inhibitor to 156.2 ± 8.0% of the negative control. The effect of miR-122 mimic and inhibitor was abolished in the cells transfected with vector plasmid or mutant reporter plasmid, and the mimic-negative control and inhibitor-negative control had no significant effect on luciferase activity, indicating that miR-122 suppressed gene expression through miR-122 binding sequence at the 3′UTR of Bcl-w mRNA (Figs. 4C, 4D).

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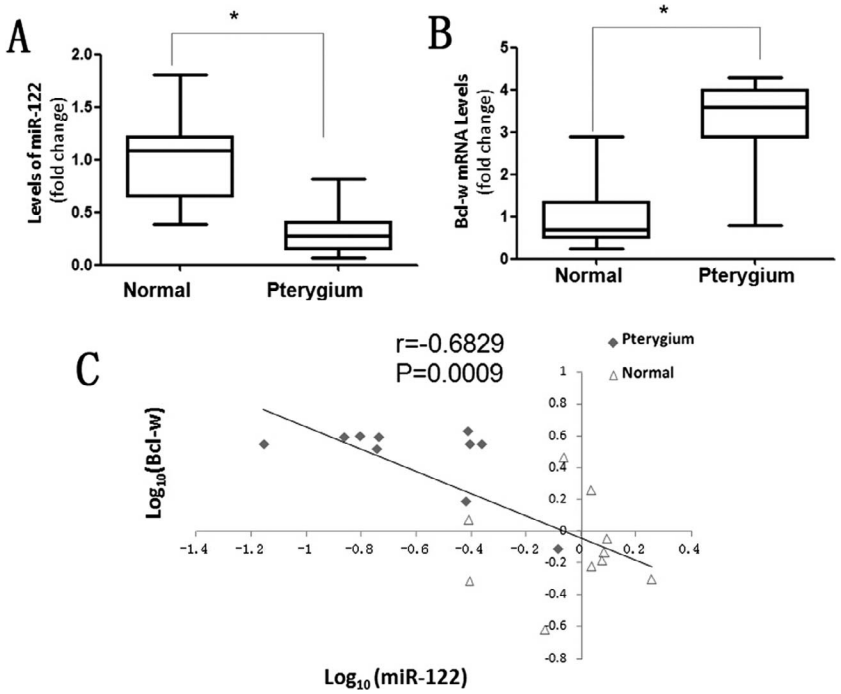

FIGURE 3. The Bcl-w mRNA expression was inversely correlated with the miR-122 expression in pterygium tissues and conjunctival tissues. (**A**, **B**) Relative expression levels of miR-122 (**A**) and Bcl-w mRNA (**B**) were measured by qRT-PCR in total RNAs extracted from 10 pterygium tissues and 10 normal conjunctival tissues. $^*P < 0.05$ compared with normal conjunctiva. (**C**) The level of miR-122 exhibited a statistically significant inverse correlation with the level of Bcl-w mRNA (Pearson's correlation: $r = -0.683$, $P < 0.05$).

MiR-122 Suppressed the Expression of Bcl-w in Pterygium Epithelial Cells

We next examined the impact of miR-122 levels on Bcl-w expression by transfecting pterygium epithelial cells with miR-122 mimics, inhibitors, or the corresponding negative controls. Transfection with miR-122 mimic (50 nM) led to a 10.2 ± 0.6-fold increase in the miR-122 level, in contrast, transfection with miR-122 inhibitor (100 nM) decreased the miR-122 level to

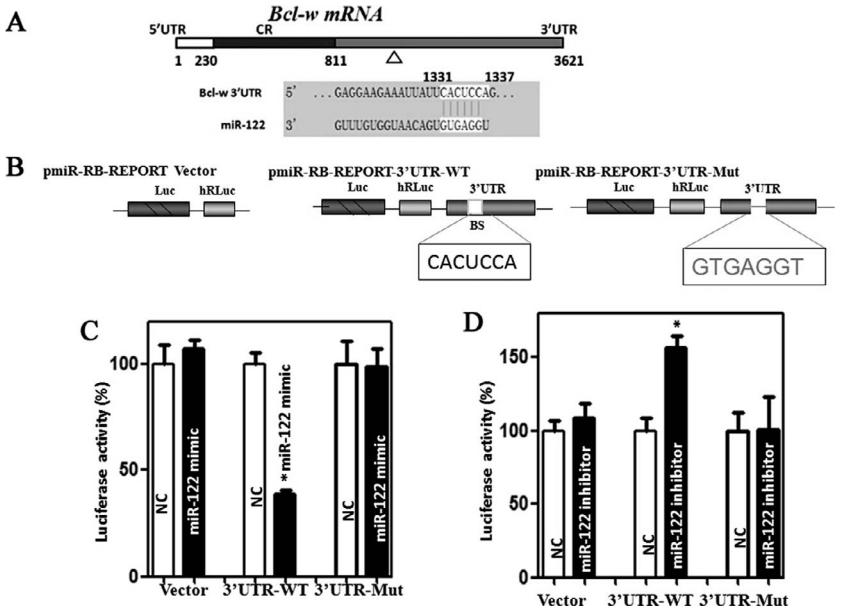

FIGURE 4. Bcl-w is a target of miR-122. (**A**) Schematic representation of Bcl-w mRNA depicting predicted target sites for miR-122 in the 3′-UTR. (**B**) Schematic representation of the vector plasmid, Bcl-w-3′-UTR reporter plasmid and its mutant form. A mutation was generated in the Bcl-w-3′-UTR sequence in the complementary site for the seed region of miR-122 as indicated. (**C**) Pterygium epithelial cells were cotransfected with 50 nM miR-122 mimic or 100 nM miR-122 inhibitor or corresponding negative controls (NC) and with 200-ng/mL vector plasmid or Bcl-w 3′-UTR reporter plasmid or its mutant form. Luciferase activity was detected 48 hours after transfection. The Renilla luciferase signal was normalized to the firefly luciferase signal. The results were expressed as fold change relative to the negative control. Values are mean ± SD from three separate experiments. $^*P < 0.05$ compared with negative controls.

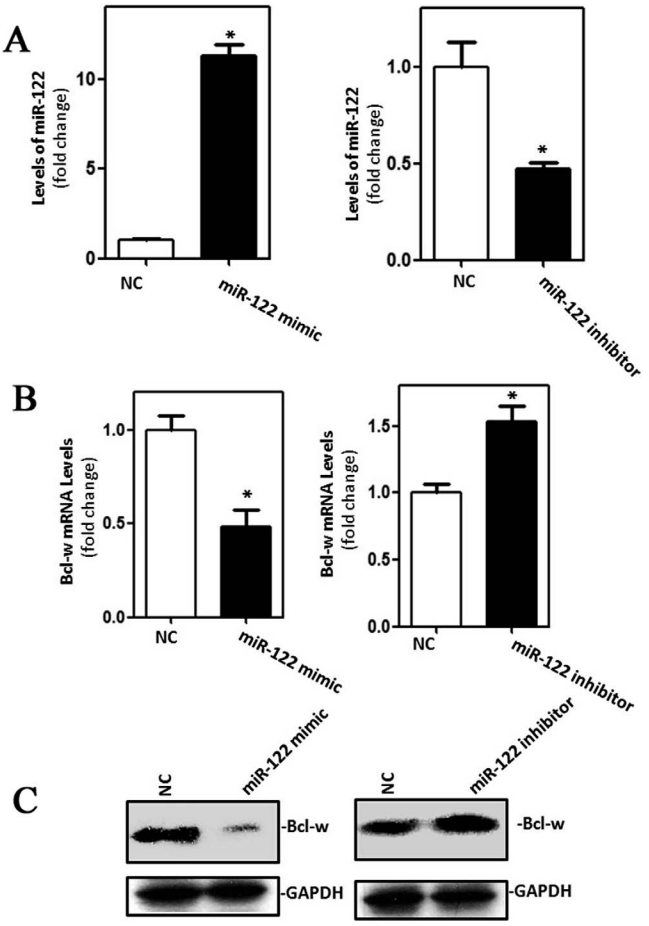

FIGURE 5. MicroRNA-122 repressed Bcl-w mRNA and protein expression in pterygium epithelial cells. (**A**) Levels of miR-122 in cells transfected with miR-122 mimic (*left*), miR-122 inhibitor (*right*), or corresponding negative controls (NC) as measured by qRT-PCR analysis. (**B**) Levels of Bcl-w mRNA as examined by qRT-PCR analysis after transfection with miR-122 mimic (*left*), miR-122 inhibitor (*right*), or corresponding NC. (**C**) Changes in Bcl-w protein expression after transfection with miR-122 mimic (*left*), miR-122 inhibitor (*right*), or corresponding NC. Whole-cell lysates were prepared for Western blotting; equal loading was monitored by assessing GAPDH levels. Values are mean ± SD from three separate experiments. $^*P < 0.05$ compared with cells transfected with NC.

47.2 ± 3.1% of the control (Fig. 5A). Transfection with miR-122 mimic decreased the Bcl-w mRNA to 48.2 ± 9.4% of the negative control and transfection with miR-122 inhibitor increased the Bcl-w mRNA by 51.0 ± 10.3% (Fig. 5B). With Western blot experiments, we also found the Bcl-w protein expression was significantly reduced by transfection with miR-122 mimic, and in contrast, was significantly increased by transfection with miR-122 inhibitor (Fig. 5C). The transfection with mimic-negative control and inhibitor-negative control had no significant effect on miR-122 levels, Bcl-w mRNA, and Bcl-w protein levels, respectively ($P > 0.05$)

miR-122 Regulated Pterygium Epithelial Cells Apoptosis via Targeting Bcl-w Expression

Because our abovementioned results showed the miR-122 expression was significantly decreased in pterygium compared with normal conjunctival tissues, we investigated the effect of miR-122 downregulation on the apoptosis of pterygium epithelial cells in vitro. We transfected pterygium epithelial cells with miR-122 inhibitor with or without Bcl-w specific siRNA or control siRNA followed by treatment with TNFα plus CHX for 4 hours. Cell apoptosis were determined with TUNEL staining (Fig. 6A).

The results showed the apoptosis rate was decreased from 35.7 ± 5.2% in the control group to 15.5 ± 3.9% in the miR-122 inhibitor transfected group, and it was increased to 33.5 ± 6.0% by cotransfection with Bcl-w siRNA. However, cotransfection of control siRNA showed no obvious effect on cell apoptosis (Fig. 6B). We also investigated the caspase activity by using a fluorimetric assay. The results showed the caspase 3/7 activity was significantly decreased by transfection of miR-122 inhibitor to 58.0 ± 7.5% of the TNFα/CHX-treated group, and it was significantly increased by cotransfection of Bcl-w specific siRNA to 86.6 ± 9.0% of the TNFα/CHX-treated group. Cotransfection of control siRNA had no significant effect on caspase 3/7 activity (Fig. 6C). These results indicated inhibition of miR-122 reduced pterygium epithelial cell apoptosis via targeting Bcl-w expression.

DISCUSSION

Pterygium, which is believed to be closely associated with ultraviolet exposure, is a common ocular surface disease, especially in the regions with intensive sunlight radiation. To date this lesion has no treatment but surgical excision. In the present study, we used microarray methods to find the differently expressed miRNAs in pterygium compared with normal conjunctiva and also explored the potential mechanism involved in the miRNA action, which might be helpful to understand the pathogenesis of pterygium.

A previously published article by Engelsvold et al.[18] reported the miRNAs expression profiles in pterygium. However, we noticed that there existed great difference in the microRNA profile between that study and ours. We think many factors might be responsible for such a difference. Firstly, the included subjects were different in ethnicity. The population in our study is Han ethnicity, which was demonstrated to be associated with a higher risk of pterygium compared with another ethnicity.[19] Larrayoz et al.[20] also revealed that genetic factors might contribute to the racial disparities in the ptergyium patients in the treatment response. Secondly, there exists great difference in the living environment of the included subjects. The subjects in our study were all from Guangzhou, China, a city at low latitude with high PM2.5 pollution and intense ultraviolet radiation. Thirdly, different tissue specimens were used as control in these two studies. We used the normal conjunctival tissue from cataract patients as control while Engelsvold et al.'s[18] study used the adjacent conjunctiva from the same pterygium patients as control. We think the adjacent conjunctiva tissue from pterygium patients might not be normal at the transcriptomic level, although it is normal in appearance. Finally, the microarray detection platform also might contribute to the difference in the results.

MicroRNA-122 is the most abundant miRNA in the liver accounting for approximately 70% of the total miRNA population and plays an important role in many aspects of liver physiology.[21–23] Pathogenic repression of miR-122 has been demonstrated to be associated with various liver diseases including nonalcoholic steatohepatitis, liver cirrhosis, and hepatocellular carcinoma.[24–26] Although miR-122 has been described as a liver-specific miRNA, many studies have investigated the presence and function of miR-122 in other tissues.[27–30] MiR-122 has been shown to stimulate apoptosis in hepatocyte and breast cancer cells, but block apoptosis in malignant T-lymphocytes, indicating the biological function of

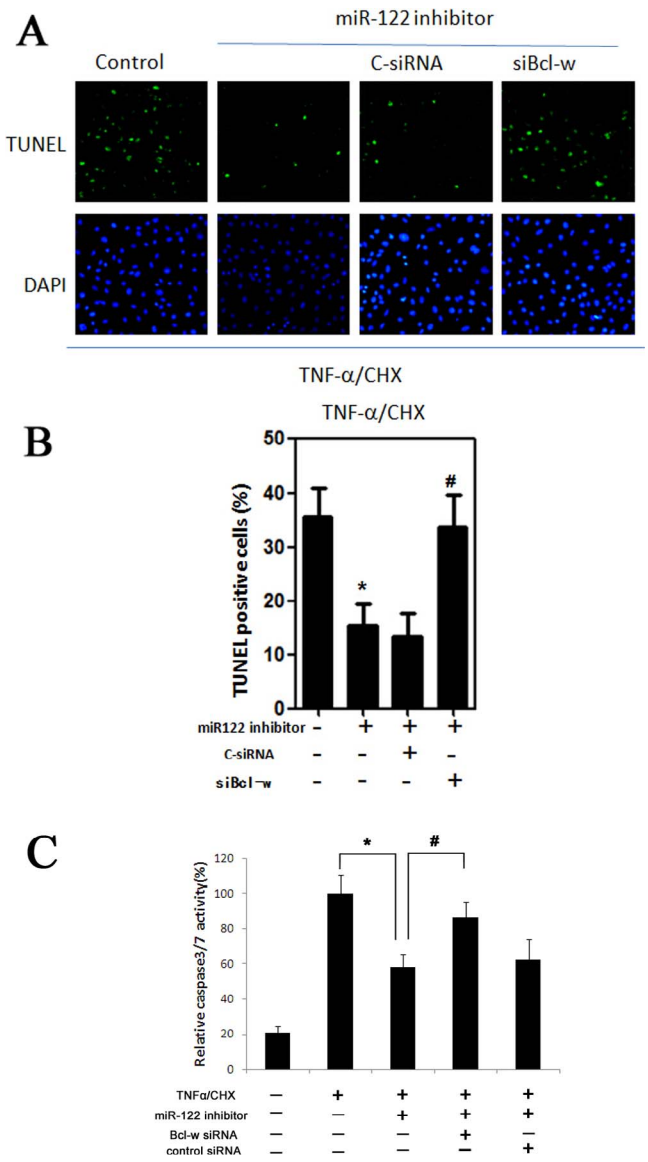

FIGURE 6. The effect of miR-122 on TNFα/CHX induced apoptosis in pterygium epithelial cells. Pterygium epithelial cells were transfected with the miR-122 inhibitor (100 nM) with or without Bcl-w siRNA/control siRNA (50 nM) as indicated followed by stimulation with TNFα (20 ng/mL) plus/CHX (25 μg/mL) for 4 hours. (**A**) Tumor necrosis factor α/CHX-induced apoptosis of pterygium epithelial cells was determined by TUNEL/DAPI staining. (**B**) The percentage of apoptotic cells was calculated and plotted. Values are mean ± SD from three separate experiments. $^{*}P < 0.05$ compared with the control; $\#P < 0.05$ compared with the cells stimulated by only miR-122 inhibitor. (**C**) Caspase 3/7 activity in pterygium epithelial cells was determined with Caspase-Glo 3/7 assay kit (Promega). Values are mean ± SD from three separate experiments. $^{*}P < 0.05$, comparison between the two indicated groups; $\#P < 0.05$, comparison between the two indicated groups.

miR-122 is highly tissue specific.[31] In this study, for the first time we identified that miR-122 was remarkably reduced in pterygium compared with normal conjunctiva and we also investigated the potential target of miR-122 in cultured pterygium epithelial cells.

Bcl-w is an important antiapoptotic member of the bcl-2 family, which is characterized by its ability to regulate apoptosis under a broad range of physiological and experimentally induced conditions. Increased Bcl-w expression has been found in various types of cancers, and depletion of Bcl-w has been proven to sensitize cells to apoptosis.[32–35] Bcl-w suppresses apoptosis by interacting directly with proapoptotic members to block their apoptotic activities.[36] Bcl-w expression is found in a wide range of tissues, particularly in the brain, spinal cord, colon, testes, most hematopoietic cells, and fibroblasts.[37] Many studies have shown the expression of apoptosis-related protein in pterygium, such as bcl-2, bak, p53, survivin, and so on, and the results support the possible association between upregulation of antiapoptotic protein and the pterygium.[4–6] This is the first study to observe the expression of Bcl-w in pterygium. A previous study has shown that apoptotic cells were seen throughout the entire width of the epithelium in normal conjunctival specimens. However, in the pterygium specimen, apoptosis was mainly confined to the basal layer but not found in the superficial layer of the epithelium.[4] We supposed the increased expression of Bcl-w in the superficial layer of epithelium in pterygium might be associated with the abnormal apoptosis. With TUNEL assay and caspase activity assay, our results demonstrated the miR-122 could regulate pterygium epithelial cells apoptosis via targeting Bcl-w.

The miR-122–mediated repression of Bcl-w expression is of biological significance, because it plays an important role in the regulation of conjunctival epithelial cells apoptosis and is thus implicated in maintaining homoeostasis of the conjunctival tissue. Apoptosis occurs in the normal conjunctiva and it maintains the critical balance in cell number. In pterygium tissues, miR-122 downregulation leads to increased expression of Bcl-w, which protects pterygium epithelial cells from apoptosis. It is possible that increased Bcl-w might have effects other than regulation of apoptosis in pterygium pathogenesis. Bcl-w is demonstrated to induce the expression of MMPs in gastric cancer and promote the invasiveness.[38] Because MMPs also play an important role in the development of pterygium,[2,3] we hypothesize that the increased expression of Bcl-w might contribute to pterygium invasiveness by increasing the MMPs secretion, which should be further investigated. A recent study by Wu et al.[39] revealed miR-221 might result in p27Kip1 gene downregulation and influence pterygium pathogenesis. Whether miR-122 interacts with other miRNAs such as miR-221 in the pathogenesis of pterygium remains unclear.

In summary, the results of this study show that decreased expression of miR-122 in pterygium might result in abnormal cell apoptosis via its regulation of the expression of Bcl-w, and subsequently contribute to the development of pterygium. These findings help to explore the mechanisms in the pathogenesis of pterygium and indicate miR-122 might be a novel therapeutic target for the treatment of pterygium.

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