miR-17-5p regulates the proliferation and apoptosis of human trabecular meshwork cells by targeting phosphatase and tensin homolog

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Abstract. Glaucoma is one of the leading causes of blindness. Previous studies have indicated that the oxidative stress-induced apoptosis of trabecular meshwork cells (TMCs) may serve a key role in the pathogenesis of glaucoma, and that micro RNA(miR)-17-5p may be involved in this process. However, the specific mechanisms require further investigation. The aim of the present study was to investigate the effects of miR-17-5p on the proliferation and apoptosis of human TMCs (HTMCs) in response to oxidative stress. It was observed that exposure to H2O2 induced a significant decrease in the proliferation and a marked increase in the apoptosis of HTMCs. H2O2 exposure also suppressed the expression of miR-17-5p and promoted the expression of phosphatase and tensin homolog (PTEN). Furthermore, transient overexpression of miR-17-5p induced a significant increase in the proliferation and a significant decrease in the apoptosis of HTMCs by affecting the expression of PTEN, and the apoptosis-related proteins B-cell lymphoma-associated X protein (Bax), B-cell lymphoma-extra large (Bcl-xl) and B-cell lymphoma-2 (Bcl-2). However, knockdown of miR-17-5p demonstrated the opposite results. The results of a dual luciferase reporter assay demonstrated that PTEN may be a direct target of miR-17-5p. In conclusion, miR-17-5p was downregulated in HTMCs under oxidative conditions, and miR-17-5p may regulate the apoptosis of HTMCs by targeting PTEN. These results provide a novel theoretical basis and potential therapeutic target for the treatment of glaucoma.

Introduction

Glaucoma is a common ophthalmic disease, and it is the second leading cause of blindness worldwide (1). It is estimated that by 2020, the number of patients with glaucoma will reach approximately 80 million (2). Glaucoma can be divided into open-angle glaucoma (OAG) and angle-closure glaucoma (ACG), and in Western countries, approximately 75% of glaucoma patients are diagnosed with OAG (3), while in Asian countries, ACG is more prevalent than OAG (4). In 2010, 86.5% of ACG patients were resident in Asia, and the largest number of patients affected by ACG was in China (47.5% of the total) (5). In recent years, increasing evidence has indicated that the oxidative stress-induced senescence of trabecular meshwork cells (TMCs) may play a key role in the occurrence and development of glaucoma (6,7); however, the specific mechanisms still require further investigation.

MicroRNAs (miRNAs) are short non-coding RNAs (length, ~22 nucleotides) that can bind to the 3’-UTR of target mRNAs and consequently suppress the expression of target genes. miRNAs can regulate a variety of biological activities, including embryonic development, angiogenesis, cell differentiation, proliferation and apoptosis (8-10). With the development of next-generation sequencing and bioinformatics methods, differentially expressed miRNAs that are potentially involved in the pathogenesis of diseases, including cancers (11,12) and glaucoma (13-15), have been successfully identified. The targets of these miRNAs, as well as related signaling pathways, have also been predicted. miR-17-5p is located on human chromosome 13q31, and it belongs to the miR-17-92 cluster (16). miR-17-5p is up-regulated in many tumor types and participates in the pathogenesis of different cancers as an oncogene (17-19). Besides cancer-related studies, miR-17-5p has been reported to promote oxidative stress-induced apoptosis of cardiomyocytes in ischemia/reperfusion-induced cardiac injury animal models (20). It has also been observed that knockdown of miR-17-5p can restore the expression of the very-low-density lipoprotein receptor and alleviate the symptoms of atherosclerosis in ApoE-/- mouse models (21). It has been observed that miR-17-5p is down-regulated in the trabecular meshwork under oxidative conditions (4). Previous studies have indicated that the oxidative stress-induced apoptosis of trabecular meshwork cells (TMCs) may serve a key role in the pathogenesis of glaucoma, and that miR-17-5p may be involved in this process. However, the specific mechanisms require further investigation.
stress (22); however, the specific mechanisms have not yet been investigated.

In the present study, we focused on the roles of miR-17-5p in glaucoma and its related mechanisms. Human TMCs (HTMCs) were treated with \( \text{H}_2\text{O}_2 \) to induce oxidative stress, and the effects of miR-17-5p on the behaviors of HTMCs under oxidative stress were explored. We hypothesized that miR-17-5p would regulate the proliferation and apoptosis of HTMCs by targeting phosphatase and tensin homolog (PTEN). The present study may provide a theoretical basis and novel therapeutic target for the treatment of glaucoma.

Materials and methods

**HTMC culture.** HTMCs were purchased from ScienCell Research Laboratories (San Diego, CA, USA). According to the product information, HTMCs were isolated from the juxtacanalicular and corneoscleral regions of human eyes, and characterized by immunofluorescence methods using \( \alpha \)-smooth muscle actin- and fibronectin-specific antibodies. Cells were cultured in a humidified incubator at 37°C with 5% \( \text{CO}_2 \) using Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplied with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin solution (Gibco; Thermo Fisher Scientific, Inc.). Oxidative stress was induced by treating HTMCs with DMEM that contained 300 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) (Beyotime Institute of Biotechnology, Shanghai, China) for 3-7 h. Then, the cells were harvested for future analysis.

**Cell transfection.** The miR-17-5p inhibitor and miR-17-5p mimic oligonucleotides were synthesized by GenePharma Co., Ltd., (Shanghai, China). HTMCs were cultured until confluence, trypsinized and seeded onto new six-well plates at 100,000 cells/well. Cells were then transfected with either miR-17-5p inhibitor or miR-17-5p mimic using Lipofectamine™ 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. After transfection, cells were cultured for 48 h and harvested for future analysis.

**Cell proliferation analysis.** An MTT assay was performed to determine the viability of HTMCs following different treatments. An MTT proliferation assay kit (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was used, according to the manufacturer's protocol.

**Cellular apoptosis analysis.** For cellular apoptosis analysis, HTMCs subjected to different treatments were stained using an Annexin V-FITC apoptosis detection kit (Sigma-Aldrich; Merck KGaA). The apoptosis rate of the cells in different groups was detected and analyzed using a BD FACSDiva flow cytometer (BD Biosciences, San Jose, CA, USA), according to the manufacturer's protocol.

**Reverse transcription-quantitative PCR (RT-qPCR).** Total RNA was extracted from HTMCs using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.), and reverse transcribed into cDNA using the PrimeScript™ RT Master Mix (Perfect Real Time) kit (Takara Biotechnology Co., Ltd., Dalian, China), then qPCR was performed using the SYBR ExScript RT-PCR kit (Takara Biotechnology Co., Ltd.) on an ABI 7300 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The thermocycling profiles are shown in Table I. The relative expression of PTEN in each sample was normalized to the level of GAPDH using the 2<sup>ΔΔCq</sup> method. The expression of miR-17-5p was examined using the Hairpin-it™ miRNAs qPCR Quantitation Kit (GenePharma Co., Ltd.) according to the manufacturer's protocol, and U6 (RNU6B; GenePharma Co., Ltd.) was used for normalization. Primers were synthesized by GenScript (Nanjing, China), and the sequences of the primers are shown in Table II.

**Western blot analysis.** Cells were lysed using RIPA buffer (Beyotime Institute of Biotechnology), and the concentration of total protein was measured using a BCA Kit (Beyotime Institute of Biotechnology). Next, SDS-PAGE was performed to separate the proteins, and the proteins were then transferred onto polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat milk and incubated with primary antibodies (anti-Bax, anti-PTEN, anti-Bcl-2, anti-Bcl-xL and anti-GAPDH, all purchased from Abcam, Cambridge, MA, USA) overnight at 4°C. The following day, the membranes were incubated with HRP-conjugated secondary antibodies (purchased from Abcam, Cambridge, MA, USA), then washed and incubated with the enhanced chemiluminescence reagent (Beyotime Institute of Biotechnology). Finally, the signals were detected using the ChemiDoc™ XRS+ imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Immunocytochemistry.** HTMCs were fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton X-100, then incubated with the anti-PTEN primary antibodies (anti-Bax, anti-PTEN, anti-Bcl-2, anti-Bcl-xL and anti-GAPDH, all purchased from Abcam, Cambridge, MA, USA) overnight at 4°C. The following day, the membranes were incubated with Alexa Fluor 488-conjugated secondary antibodies, then visualized using an Olympus fluorescent microscope (Olympus Corporation, Tokyo, Japan).

**Computational methods.** The target genes of miR-17-5p were predicted using online bioinformatics tools, TargetScan (http://www.targetscan.org) and miRanda (http://www.microrna.org).

**Dual luciferase reporter assay.** Fragments of either the wild-type PTEN 3'UTR (PTEN-3’UTR) or mutant PTEN 3’UTR (PTEN-MUT) region that contains the miR-17-5p binding site were synthesized and cloned into the pGL6-TA-reporter plasmid (Beyotime Institute of Biotechnology). The plasmids were transfected into 293 cells using Lipofectamine™ 3000 for 48 h, and the activity of the luciferase in each group was examined using the dual-luciferase reporter system (Beyotime Institute of Biotechnology), according to the manufacturer's protocol.

**Statistics.** All statistical analyses were conducted using SPSS v.22.0 software (IBM Corp., Armonk, NY, USA). Data are presented as the mean ± standard deviation. Two independent sample t-tests were performed for comparisons between two
groups. One-way analysis of variance followed by Dunnett’s post-hoc test was performed for comparisons among multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of oxidative stress on the proliferation and apoptosis of HTMCs in vitro. First, we investigated the effect of H$_2$O$_2$ treatment on the proliferation and apoptosis of HTMCs using MTT and flow cytometry methods. It was observed that the cell viability was significantly decreased in cells treated with H$_2$O$_2$ compared with the control group at 3, 5 and 7 h (P<0.01; Fig. 1A). Moreover, H$_2$O$_2$ treatment also induced a continuous increase in the apoptosis rate of HTMCs from 0 to 7 h (P<0.05; Fig. 1B and C).

miR-17-5p was down-regulated in HTMCs under oxidative stress in vitro. In order to explore the roles of miR-17-5p in HTMCs under oxidative stress, we compared the expression of miR-17-5p in control and H$_2$O$_2$ groups at different time points using RT-qPCR methods. As shown in Fig. 2A, H$_2$O$_2$ induced a significant decrease in the expression of miR-17-5p at 5 and 7 h (P<0.05; Fig. 2A).

miR-17-5p can regulate the proliferation and apoptosis of HTMCs in vitro. HTMCs were transfected with either miR-17-5p mimics or miR-17-5p inhibitor, and the effect of miR-17-5p on the proliferation and apoptosis of HTMCs was examined using MTT and flow cytometry methods. As shown in Fig. 2B, the expression of miR-17-5p was significantly increased in miR-17-5p mimic-transfected HTMCs and markedly decreased in miR-17-5p inhibitor-transfected HTMCs, suggesting that transfection had been successfully performed. Moreover, transient overexpression of miR-17-5p induced a significant increase in the proliferation (P<0.01; Fig. 3A and B) and a significant decrease in the apoptosis of HTMCs (P<0.01; Fig. 3C). Knockdown of miR-17-5p exhibited the opposite results. In addition, transfection of miR-17-5p mimics also induced a significant increase in the expression of Bcl-2 and Bcl-xL, and a marked decrease in the expression of Bax, while knockdown of miR-17-5p exhibited the opposite results (Fig. 4).
Figure 2. Expressions of miR-17-5p in HTMCs by RT-qPCR methods. (A) Effect of oxidative stress on the expression of miR-17-5p in HTMCs. (B) Effect of transfection of miR-17-5p mimics or inhibitor on the expression of miR-17-5p in HTMCs. *P<0.05 and **P<0.01 vs. the control group. HTMCs, human trabecular meshwork cells.

Figure 3. Effect of miR-17-5p on the proliferation and apoptosis of HTMCs in vitro. (A) Morphological feature of HTMCs in different groups by inverted microscope (scale bar, 100 µm); (B) Proliferation rate of HTMCs in different groups by MTT assay; (C) Apoptosis rate of HTMCs in different groups by flow cytometry method. *P<0.01 vs. the control group. Control, un-transfected cells; Inhibitor, miR-17-5p inhibitor transfected cells; Mimics, miR-17-5p mimics transfected cells. HTMCs, human trabecular meshwork cells.
PTEN is a direct target of miR-17-5p in HTMCs. Using online bioinformatics tools, PTEN was predicted to be a direct target of miR-17-5p. Using western blot analysis, it was observed that the expression of PTEN continuously increased from 3 to 7 h under oxidative stress (Fig. 5A), suggesting that increased PTEN may be associated with the pathogenesis of glaucoma. Moreover, transient overexpression of miR-17-5p in HTMCs induced a significant decrease in the expression of PTEN, while knockdown of miR-17-5p promoted the expression of PTEN in HTMCs (Fig. 5B and C). Finally, the results from the dual luciferase reporter assay indicated that co-transfection of miR-17-5p mimics and wild-type PTEN-3’UTR plasmids significantly decreased the activity of the luciferases. Co-transfection of miR-17-5p mimic and PTEN-MUT exhibited no effect on the activity of the luciferases (Fig. 6), suggesting that PTEN is a direct target of miR-17-5p.

Discussion

Glaucoma is a common ophthalmic disorder that can cause damage to the optic nerve and loss of vision. Increasing evidence has indicated that oxidative stress-induced senescence of HTMCs is involved in the pathogenesis of glaucoma (7). H₂O₂ is an important oxidant in the human eye. It has been demonstrated that oxidative levels significantly increase in the aqueous humor of patients with glaucoma, which means that HTMCs will also be exposed to high concentrations of H₂O₂. Thus, in previous studies, a high concentration of H₂O₂ has been used to induce oxidative stress in HTMCs, and this has now become a widely applied in vitro model in glaucoma-related studies (23,24).

In recent years, the effects of miRNAs on TMCs have been discussed in many studies (25,26). Li et al (22), performed miRNA array analysis to examine alterations in the expression levels of microRNAs in oxidative stress-induced cellular senescence in HTMCs. It was observed that the expression of miR-17-5p was significantly decreased by treatment with a high concentration of H₂O₂. However, the specific mechanisms underlying this remain unclear. In the present study, we identified that compared with untreated cells, H₂O₂ treatment induced a significant decrease in the proliferation and a marked increase in the apoptosis of HTMCs at each time point examined (P<0.05). Moreover, H₂O₂ treatment also induced a significant decrease in the expression of miR-17-5p, which was consistent with the finding from Li et al. These results indicate that miR-17-5p is down-regulated in HTMCs under oxidative stress, and miR-17-5p may participate in the mechanism of H₂O₂-induced increase in apoptosis and decrease in the proliferation of HTMCs.
The present study has certain limitations. We only performed cellular experiments, and the expression levels of miR-17-5p in tissue samples from patients and normal controls should be evaluated to confirm our findings from the clinical perspective. However, due to ethical issues, it is difficult to obtain the trabecular meshwork of healthy volunteers; thus, in vivo studies using animal models could be a better option.

In conclusion, our data demonstrated that miR-17-5p is down-regulated and PTEN is up-regulated in HTMCs under oxidative conditions. We also identified that miR-17-5p can regulate the proliferation and apoptosis of HTMCs through targeting PTEN. Our data indicated that miR-17-5p has the potential to become a novel therapeutic target for the treatment of glaucoma.

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Availability of data and materials

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

Authors’ contributions

FZ designed the study and wrote the majority of the manuscript. XW performed most of the experiments and wrote part of the manuscript. ZL and JB performed some of the experiments. WS performed some of the statistical analysis.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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