

Bioinformatics and *in vitro* study reveal the roles of microRNA-346 in high glucose-induced human retinal pigment epithelial cell damage

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Received: 2023-04-05

Accepted: 2023-07-05

Abstract

• **AIM:** To study microRNAs (miRNAs) and their potential effects in high glucose-induced human retinal pigment epithelial cell damage.

• **METHODS:** We screened the GSE52233 miRNA expression dataset for differentially expressed miRNAs (DEMs). The target genes of the top 10 DEMs were predicted using miRWalk 2.0 database, followed by function enrichment and protein-protein interaction analysis. miRNA expression was determined in the human retinal pigment epithelial cell line ARPE-19 treated with high glucose (HG) by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Cell proliferation was determined using cell counting kit (CCK)-8 assay. Cell cycle, apoptosis, and reactive oxygen species (ROS) levels were determined by flow cytometry. The direct interaction between miRNA and targets was validated using dual-luciferase reporter assay.

• **RESULTS:** Thirty-nine DEMs were screened, and we predicted 125 miRNA-mRNA pairs for the top 10 DEMs, including 119 target genes of seven DEMs such as miR-346, which was upregulated in diabetic retinopathy (DR). miR-346 target genes were substantially enriched in the regulation of intracellular transport and retinoic acid-inducible gene I (RIG-I)-like receptor signaling pathway. Expression of three upregulated and downregulated

miRNAs were verified by qRT-PCR in HG-treated ARPE-19 cells. Expression of miR-346 was elevated in HG treated ARPE-19 cells in a dose-dependent manner. HG inhibited cell proliferation and induced apoptosis, which were partly reversed by transfecting an miR-346 inhibitor, which even decreased the ROS levels elevated due to HG. Argonaute 2 (AGO2) was a target of miR-346.

• **CONCLUSION:** miR-346 is a key miRNA and plays an important role in HG-induced damage in human retinal pigment epithelial cells.

• **KEYWORDS:** miRNA; miRNA-346; ARPE-19; bioinformatics analysis

DOI:10.18240/ijo.2023.11.04

Citation: Li P, Wang L, Liu Q, Du ZJ. Bioinformatics and *in vitro* study reveal the roles of microRNA-346 in high glucose-induced human retinal pigment epithelial cell damage. *Int J Ophthalmol* 2023;16(11):1756-1765

INTRODUCTION

Diabetes is a public health issue with an estimated 415 million patients globally in 2015 and is projected to affect 642 million people by 2040^[1]. Diabetic retinopathy (DR), a major microvascular complication of diabetes, normally characterized by abnormal retinal microcirculation, affects a third of the diabetic patients and is the main cause of irreversible blindness in adults^[2]. It is associated with a poor life quality and increases the risk of other complications and mortality^[3-5]. Inflammatory processes play important roles in the pathogenesis of DR, which has been enforced by the clinical application of dexamethasone^[6]. There are three strategies to prevent blindness induced by DR. While primary prevention requires the prevention or delay of its onset, secondary prevention is to delay the progression of DR. Lastly, non-invasive laser photocoagulation and invasive ocular surgery are the treatment options. However, anti-vascular endothelial growth factor (VEGF) is also increasingly used to treat vision-damaging DR^[7]. Therefore, detailed studies of its molecular mechanisms are essential to provide a theoretical framework for research on DR treatment.

As we know, blood-retinal barriers (BRB) is a physiologic barrier that maintain the structural and functional integrity of the retinal tissues^[8]. The BRB composed of inner and outer components. The inner BRB is formed mainly by the tight junctions of retinal endothelial cells, and outer BRB is formed by the tight junctions of retinal pigment epithelial (RPE) cells^[9]. Alterations of both inner and outer BRB play an important role in the development of retinal diseases^[8]. RPE cells were previously believed unrelated to DR. However, increasing evidence suggests that all types of retinal cell are affected by diabetes^[10], including the degeneration or dysfunction of the RPE^[11-13]. In diabetic retina, cellular changes in the RPE occur in the early stage of DR, Samuels *et al*^[11] used three mouse models to compare the time course of RPE involvement in type 1 and type 2 diabetes, and all mouse models showed altered RPE function accompanied with the onset of hyperglycemia. Recent studies have emphasized the importance of RPE in DR^[8,14]. For example, RPE contribute to the development of DR probably by promoting the retinal vascular alterations in the early stages of the retinopathy^[10]. Additionally, there have been a substantial of studies focusing on the inner retina in DR, and studies on outer retina is still limited.

Previous studies have shown that the occurrence of DR is related to abnormal epigenetic regulation^[15-17]. In eukaryotic organisms, miRNA, an important component of epigenetics, is a highly conserved class of non-coding RNAs, which are about twenty-two nucleotides long. Endogenous miRNAs can regulate gene expression at the transcriptional level through specific interactions with target gene sequences and participate in many biological processes such as cell proliferation, differentiation and apoptosis^[17-18]. miRNAs are associated with DR microvascularization, and differentially expressed miRNAs (DEMs) in DR have been identified^[19]. miR-15b has been reported to inhibit angiogenesis by targeting VEGF in proliferative DR^[20]. miR-451a mediates the proliferation and migration of RPE cells in proliferative DR by regulating mitochondrial function^[21]. Previous studies have suggested that miRNAs could be potential biomarkers for DR therapeutic strategies^[22-23]. For instance, diabetes could induce the high expression of miR-21 in retina, and inhibition of miR-21 attenuated retinal neovascularization and inflammation, indicating that miR-21 might be a therapeutic target in DR^[24]. A previous study identified 8 dysregulated miRNAs and their key targets using *in silico* method and were successfully confirmed using *in vivo* method^[25], suggesting the feasibility of identifying miRNAs using bioinformatics methods. Therefore, we screened key dysregulated miRNAs in DR using bioinformatics methods based on the miRNA expression profiling in the Gene Expression Omnibus (GEO) database.

The target genes of dysregulated miRNAs were predicted, followed by functional enrichment analysis and protein-protein interaction (PPI) analysis to further study the role of dysregulated miRNAs in DR. In addition, we investigated the effect of miR-346 in high glucose (HG)-treated human retinal pigment epithelial cells.

MATERIALS AND METHODS

Data Access The microarray dataset of GSE52233 was downloaded from the GEO database (<http://www.ncbi.nlm.nih.gov/geo/>), which contained the miRNA expression data of age-matched human autopsy normal ($n=6$) and DR ($n=3$) central corneas. The sequencing platform was GPL8786 [miRNA-1] Affymetrix Multispecies miRNA-1 Array. All methods were carried out in accordance with relevant guidelines and regulations.

Differential Expression Analysis The miRNA data from downloaded CEL files were processed using the R package of Affymetrix (Version 1.50.0, <http://www.bioconductor.org/packages/release/bioc/html/affy.html>)^[26]. The procedure included background correction and normalization. Additionally, expression calculations were performed using the robust multi-array average (RMA) method. The annotation file of the platform was used to annotate the probes for the miRNA chip, and the probes of non-human miRNAs were removed^[27-28]. The empirical Bayes linear model in the limma package was used to analyze DEMs between DR and control (normal). The criteria for differential expression were set as follows: $P < 0.05$, $|\log_2FC| > 0.585$ (fold change > 1.5 or fold change < 1.5).

Prediction of Target Genes of DEMs The target mRNAs of the DEMs (top five upregulated and top five downregulated DEMs) were predicted using miRWalk 2.0^[29-30] (<http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/>). The miRNA-mRNA pairs were included only when they appeared in all of the following twelve databases: miRWalk, microT v4, miRanda, miRBridge, miRDB, miRMap, miRNAMap, Pictar2, PITA, RNA22, RNAhybrid, and Targetscan. Cytoscape software (Version 3.4.0), which were used to visualize the miRNA-mRNA regulation network, and the CytoNCA^[31] plugin (Version 2.1.6, <http://apps.cytoscape.org/apps/cytonca>) was used to determine the connectivity degree of each node by analyzing the topological properties.

Function Enrichment Analysis To explore the functions of the DEMs, function enrichment analysis was performed for the target mRNAs of the DEMs. The hypergeometric distribution test of ClusterProfiler in R package was used to analyze the biological processes from Gene Ontology^[32] and Kyoto Encyclopedia of Genes and Genomes (KEGG)^[33] pathways. Statistical significance was set at $P < 0.05$.

PPI Network Analysis STRING database^[34] (Version: 10.0, <http://www.string-db.org/>) was used to analyze the interactions among the target mRNAs of the DEMs with PPI score ≥ 0.4 . Cytoscape software (Version 3.4.0) was used to visualize the PPI network. The CytoNCA^[31] plug-in was used to analyze the topological properties of each node in the PPI network.

Cell Culture The human retinal pigment epithelial cell line ARPE-19 was purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences, China. The cells were cultured at 37°C in a humidified incubator with 5% carbon dioxide and were maintained in basic DMEM (Catalog No.8117225, Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Catalog No.10099-141, Gibco, Grand Island, NY, USA) and 1% penicillin-streptomycin (Catalog No.BS734, Sangon biotech, Shanghai, China).

For HG treatment, ARPE-19 cells were treated with different concentrations of glucose (7.5, 17.5, 25, 50, 100, and 200 mmol/L; Catalog No.14431-43-7, Sinopharm, China) for 24, 48, and 72h. Cell viability was assessed, and 25 mmol/L and 200 mmol/L HG treatment for 48h were used in the following experiments. HG treatment (7.5 mmol/L) was used as a control.

Real-time Polymerase Chain Reaction The TRIzol reagent kit (Catalog No.15596018, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) was used to isolate total RNA according to the manufacturer's instructions. Total RNA (1 μg per sample) was used for the reverse transcription reaction with the PrimerScript reverse transcription reagent kit (Catalog No.RR036A, Takara, Shiga, Japan) according to the manufacturer's protocol. After reverse transcription, the Power SYBR Green PCR MasterMix (Applied Biosystems, Foster City, CA, USA) was used for real-time polymerase chain reaction (RT-PCR), which was performed in an ABI 7500 thermocycler (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. Each sample contained three replicates. The data were analyzed and calculated according to the cycle threshold values.

Transfection ARPE-19 cells were seeded onto 6-well plates at a density of 3×10^5 cells/well. After overnight incubation, the hsa-miR-346 inhibitor or negative control (NC) sequence was transfected into cells using Lipofectamine 2000 reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). After 24, 48, and 72h, the cells were harvested for subsequent experiments.

Cell Viability Detection A cell counting Kit (CCK-8, Catalog No. C0039, Beyotime, Shanghai, China) based on WST-8 was used to detect cell viability and cytotoxicity. After overnight incubation, approximately 4×10^3 ARPE-19 cells seeded in a 96-well plate were subsequently treated with different concentrations of glucose (7.5 mmol/L for the blank group,

25 mmol/L for the HG group). After 24, 48, and 72h of cell culture, the medium in the wells was discarded and 100 μL CCK-8 (5 mg/mL) was added to each well to a final concentration of 10% and thoroughly mixed for an additional 2h of incubation. The absorbance of formazan dye in each well was measured using a microplate reader (Thermo Multiscan MK3; Thermo Fisher Scientific, Waltham, MA, USA) at 450 nm.

Flow Cytometry Analysis Fluorescence-activated cell sorting (FACS) was performed to detect apoptosis, cell cycle profiling, and ROS activity. For apoptosis detection, four different groups were formed: 1) ARPE-19-blank (ARPE-19 cells without treatment); 2) ARPE-19+HG (ARPE-19 cells treated with 25 mmol/L HG); 3) ARPE-19-NC+HG, (ARPE-19 cells treated with hsa-miR-346 NC and 25 mmol/L HG); 4) ARPE-19-inhibitor+HG (ARPE-19 cells treated with hsa-miR-346 inhibitor and 25 mmol/L HG). Cells were then harvested and stained with FITC-Annexin V and propidium iodide (PI, 36 $\mu\text{g}/\text{mL}$; Catalog No.P4170, MilliporeSigma, St. Louis, MO, USA) with RNase (10 $\mu\text{g}/\text{mL}$; Catalog No.RNASEA-RO, MilliporeSigma, St. Louis, MO, USA) at 25°C for 15min. Cell apoptosis was measured as the percentage of FITC+ and PI-cell populations using FACS.

For cell cycle detection, cells were harvested and stained with PI (36 $\mu\text{g}/\text{mL}$; Catalog No.P4170, MilliporeSigma, St. Louis, MO, USA) with RNase (10 $\mu\text{g}/\text{mL}$; Catalog No.RNASEA-RO, MilliporeSigma, St. Louis, MO, USA) at 25°C for 15min. The cell cycle was analyzed by PI staining data from FACS.

For ROS detection, the ROS assay kit (Catalog No.S0033, Beyotime, Shanghai, China) was used. In details, ARPE-19 cells transfected with miRNA NC and miRNA inhibitor were pre-incubated for 48h. DCFH-DA was then added to four different groups of cells to a final concentration of 10 mmol/L and incubated at 37°C for 30min in the dark. The cells were subsequently washed with PBS and adjusted to a concentration of 1×10^6 cells/mL for FACS analysis under the condition that the excitation and emission wavelengths were 488 and 525 nm, respectively. Data were analyzed using ModFitLT software (Verity Software House, Topsham, ME, USA). Representative data from three independent experiments with similar trends are shown.

Dual-luciferase Reporter Assay In order to verify the direct interaction between miR-346 and AGO2, the wild type or mutant 3'-UTR fragments of Argonaute 2 (AGO2) containing the binding sites were amplified and cloned into the pmirGLO reporter vectors (Promega Corp., Madison, WI, USA). Then, miR-346 mimic or mimic NC and pmirGLO AGO2 wild type (WT)/ mutant (MUT) were then transfected into 293T cells using Lipofectamine 2000 Reagent (Invitrogen). After 48h of transfection, luciferase activity of reporter vectors were determined, and the binding intensity between miR-346

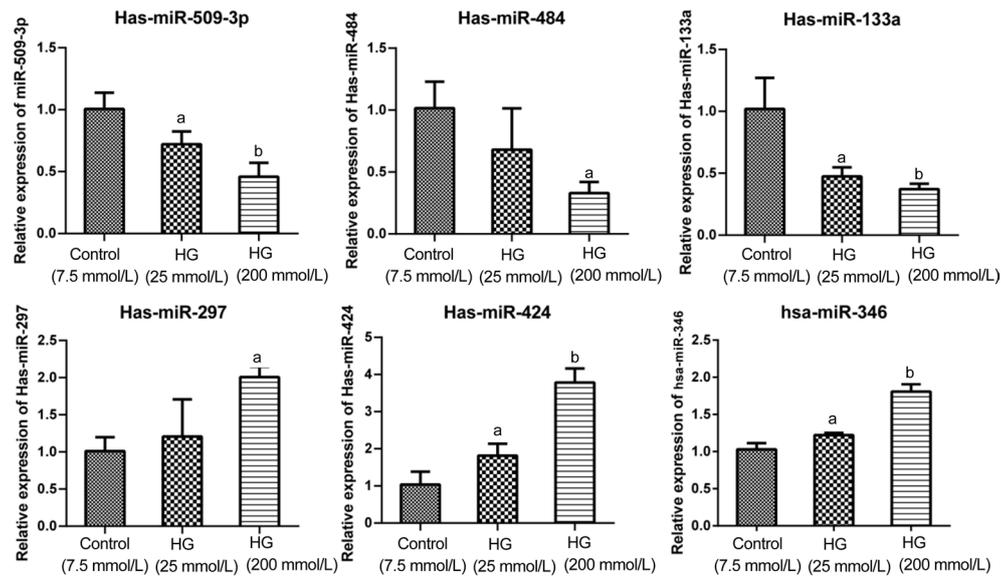


Figure 5 Expression of miRNAs validated in HG-treated ARPE-19 cells Expression of three upregulated miRNAs and three downregulated miRNAs in HG-treated ARPE-19 cells determined by qPCR. ^a $P < 0.05$; ^b $P < 0.01$, compared to control. qPCR: Quantitative polymerase chain reaction; HG: High glucose.

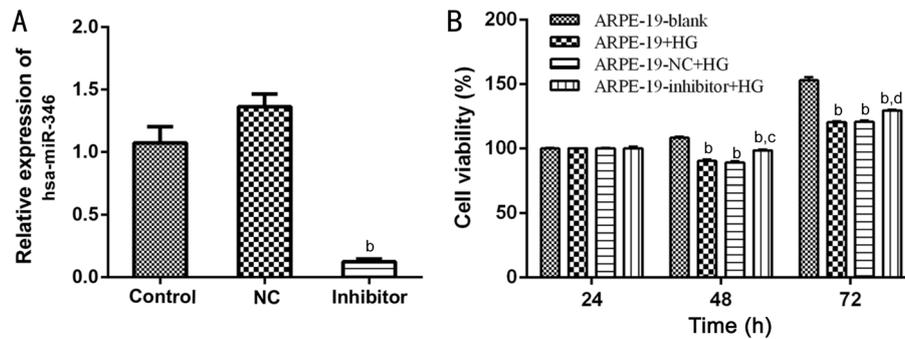


Figure 6 The effect of miR-346 on ARPE-19 cell proliferation A: Expression of miR-346 was verified to be decreased after transfecting miR-346 inhibitor. B: Cell proliferation of ARPE-19 cells determined by CCK-8 assay after HG treatment and miR-346 inhibition. ^b $P < 0.01$, compared to control, ARPE-19-blank group; ^c $P < 0.05$, ^d $P < 0.01$, compared to NC+HG group. NC: Negative control; HG: High glucose; CCK: Cell Counting Kit.

induced changes in the cell cycle could be partly reversed by inhibiting miR-346 expression (1.20 fold at G0/G1 phase, 2.24 fold at S phase, $P < 0.05$; Figure 7A). In addition, HG treatment induced ARPE-19 cell apoptosis (2.36 fold, $P < 0.01$), which was partly inhibited by inhibiting miR-346 expression (1.37 fold, $P < 0.01$; Figure 7B). These results suggest that miR-346 expression is involved in HG-induced damage in human retinal pigment epithelial cells.

Role of miRNA-346 in HG-induced Oxidative Stress HG treatment led to increased levels of reactive oxygen species (ROS, 1.64 fold, $P < 0.01$), which substantially decreased after inhibiting the expression of miR-346 (1.23 fold, $P < 0.01$, Figure 8), suggesting that miR-346 inhibition could reduce oxidative stress induced by HG.

AGO2 a Target of miR-346 In order to verify the targets of miR-346, the binding probability between miR-346 and its targets were predicted. Among the 15 targets shown in the above miRNA-mRNA interaction network, AGO2 showed highest binding probability to miR-346. Expression of AGO2

was significantly increased after inhibiting miR-346 (Figure 9A), indicating that there might be a negative regulatory between miR-346 and AGO2. Dual-luciferase reporter assay was further performed to validate the targeting relationship between miR-346 and AGO2. Luciferase activity of AGO2-WT was markedly reduced after transfection of miR-346 mimics, while luciferase activity of AGO2-MUT showed no significant changes after transfection of miR-346 mimics (Figure 9B). These results suggested that AGO2 was a target of miR-346.

DISCUSSION

DR develops at different levels in more than 40% of diabetic patients, and in about 4% of them, progresses to the proliferative type^[37], which seriously impairs visual function. The development of proliferative DR seriously damages visual function. Although surgical treatment can restore the anatomical structure of the retina, visual function remains impaired^[38-39]. Therefore, searching for biomarkers with higher sensitivity, specificity, and stability may provide potential targets for the treatment of DR.

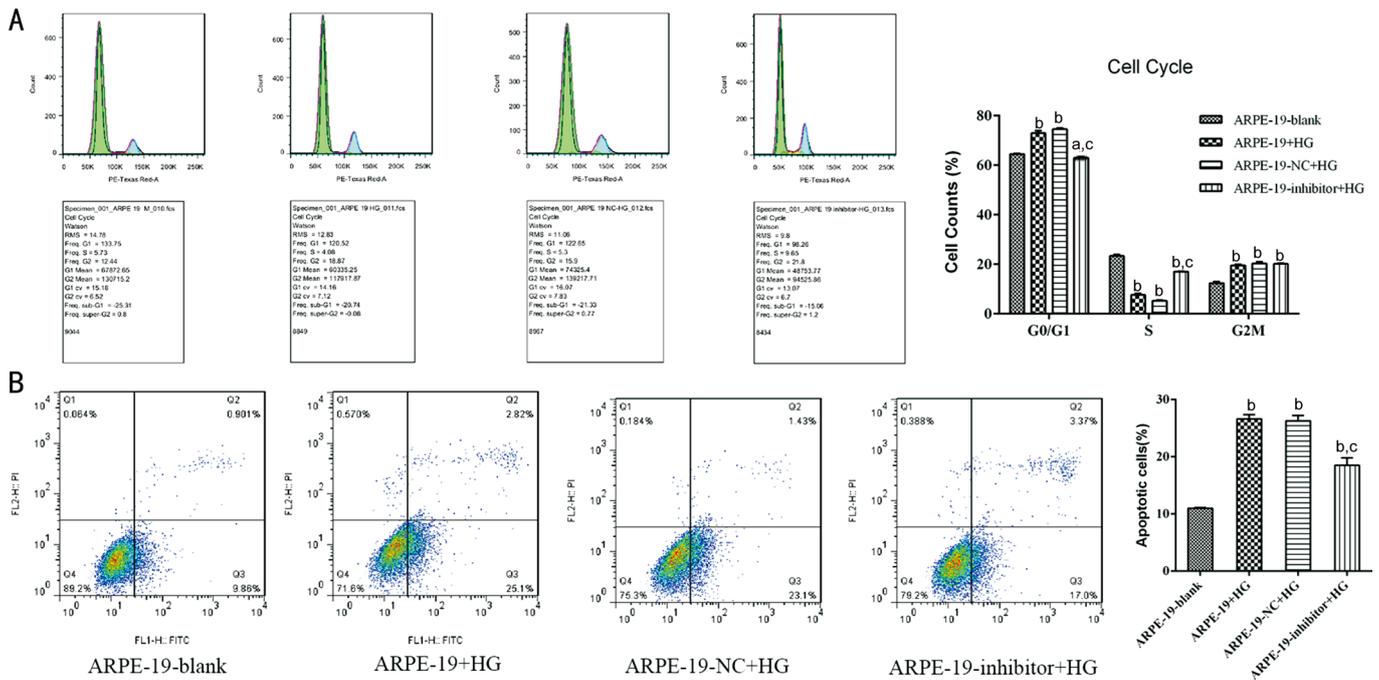


Figure 7 The effect of miR-346 on cell cycle and apoptosis of ARPE-19 cells. A: Cell cycle detection by FACS; B: Cell apoptosis detection by FACS. ^a*P*<0.05; ^b*P*<0.01, compared to ARPE-19-blank group; ^c*P*<0.001, compared to NC+HG group. HG: High glucose; FACS: Fluorescence-activated cell sorting; NC: Negative control.

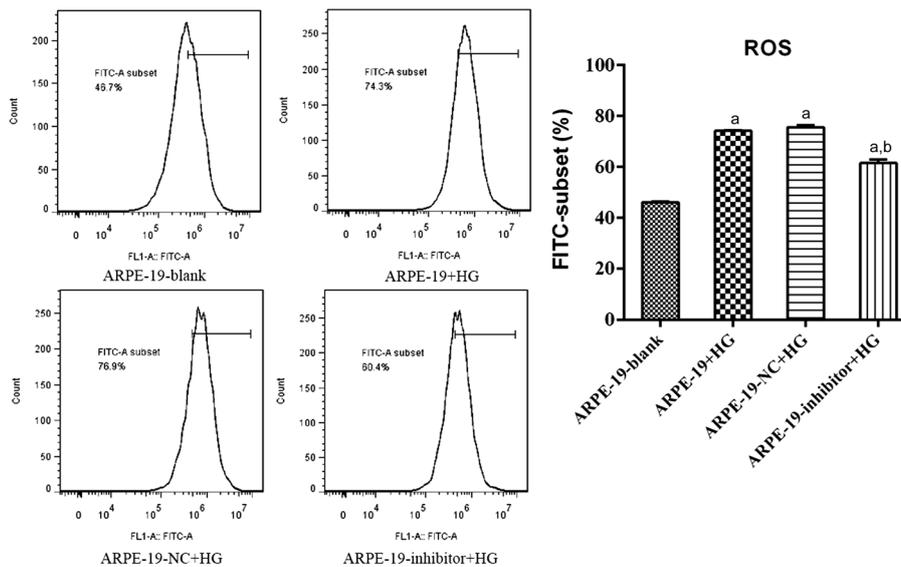


Figure 8 The effect of miR-346 on HG-induced oxidative stress ROS detection by FACS. ^a*P*<0.001, compared to blank group; ^b*P*<0.001, compared to NC+HG group. HG: High glucose; ROS: Reactive oxygen species; FACS: Fluorescence-activated cell sorting; NC: Negative control.

An increasing number of studies have demonstrated the important role of miRNAs in DR^[40-41]. For example, Bao and Cao^[42] suggested that the expression of miR-138-5p decreased in DR, and that it mediated the proliferation of retinal capillary pericytes and endothelial cells by regulating NOVA1. In this study, a total of 39 DEMs were screened for DR. Decreased expression of miR-509-3p, miR-484 and miR-133a, while elevated expression of miR-297, miR-424 and miR-346 were verified in HG treated ARPE-19 cells. The detailed roles of these miRNAs have not been reported in DR. Noticeably, miR-484 was reported to participate in apoptosis of the retinal

ganglion cells following retinal ischemia reperfusion injury^[43]. MiR-133a-3p was found to locate at the outer nuclear layer in the damaged retina^[44], and miR-424-5p was found to be up-regulated in hypoxia-induced high-altitude retinopathy cell model^[35]. Further investigations focusing on the involvements of these miRNAs in DR should be carried out. In this study, expression of miR-346 was found to increase in central corneas of DR patients, and its increased expression was verified in HG-treated ARPE-19 cells in a dose-dependent manner, suggesting the involvement of miR-346 in DR. However, there was no study to report the roles of miR-346

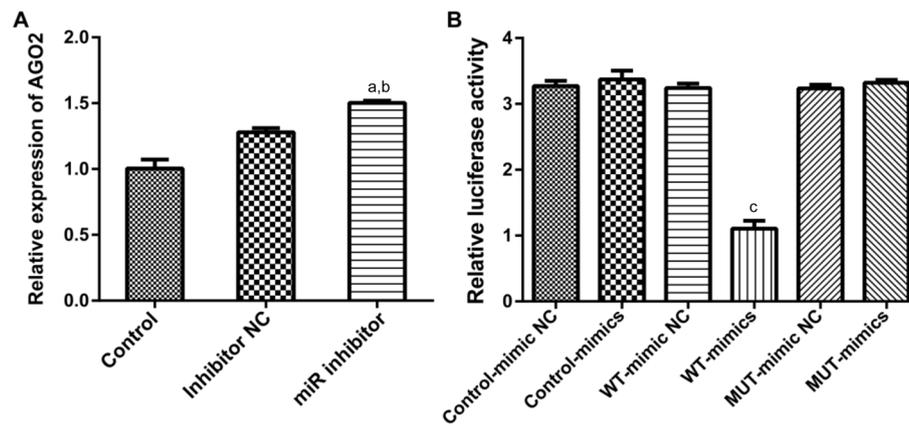


Figure 9 AGO2 was a target of miR-346 A: Expression of AGO2 after miR-346 inhibition; B: Dual-luciferase reporter assay for determining the interactions between miR-346 and AGO2. ^a $P < 0.01$, compared to control; ^b $P < 0.01$, compared to inhibitor NC; ^c $P < 0.001$, compared to WT-mimic NC. AGO2: Argonaute 2; WT: Wild type; NC: Negative control.

in DR. Here, we found that the target gene of miR-346 was mainly enriched in the regulation of intracellular transport, (RIG-I)-like receptor signaling pathway, and synaptic vesicle cycle. A study by Miao *et al*^[45] found that aberrantly hypermethylated genes that were enriched in the synaptic vesicle cycle and visual perception were involved in the pathophysiology of proliferative DR. (RIG-I)-like receptors play an important role in cytoplasmic RNA sensing, where viral RNAs are recognized and innate immune system and inflammation in cells is induced^[46]. The regulation of these signals is especially crucial to decrease inflammation in the eyes and other immune-tolerant organs^[47]. We speculated that miR-346 was involved in DR progression, probably by targeting genes associated with these signaling pathways. We further verified the targets of miR-346, and AGO2 was confirmed to be directly regulated by miR-346 in dual-luciferase reporter assay. In HG-treated ARPE-19 cells, expression of AGO2 was significantly increased after inhibiting miR-346. Reportedly, either inhibiting or overexpressing AGO2 in mouse retina led to significantly changed retinal morphological and severely damaged retinal function^[48]. This finding also proven that miR-346 may be involved in the occurrence and development of DR to a certain extent.

RPE is a monolayer of pigment epithelium cells which comprises the outer blood-retinal barrier, and represents an important site for retinopathy^[49]. The damage of HG to RPE cells has been considered as an important event in DR, and the damage was implicated with apoptosis, inflammation, and oxidation^[10]. Especially, oxidative stress has been demonstrated to be an important contributor for the etiopathogenesis of DR^[50]. ARPE-19 cells are sensitive to oxidative damage, while abundant oxidative metabolites are produced in cells after HG stimulation^[51-52]. The accumulation of oxidative metabolites may result in irreversible cytotoxic damage to ARPE-19 cells^[53]. In addition, increased free radicals may

trigger apoptosis of ARPE cells by damaging mitochondrial DNA^[54]. In order to confirm the involvement of miR-346 in the pathogenesis of DR, functional experiments were performed in HG-treated ARPE-19 cells. We found that miR-346 inhibition could alleviate HG-induced decreased cell viability and increased cell apoptosis. In addition, inhibition of miR-346 could also stabilize the increased rate of the G0/G1 phase and the decreased rate of the S phase after HG treatment. Most importantly, the increased level of ROS caused by HG could be reversed by inhibiting miR-346. These findings verified the involvement of miR-346 in the pathogenesis of DR.

To our knowledge, this was the first study to investigate the role of miR-346 in DR. However, this was just a preliminary study, and remained some limitations. The bioinformatics analysis revealed that the targeted genes of miR-346 enriched in several pathways reported to be involved in DR. Whether miR-346 affected these pathways had not been investigated in the *in vitro* experiments. This study suggested that miR-346 mediated the basic biological processes in HG-induced damage of retinal pigment epithelial cell, such as cell proliferation, apoptosis and oxidative stress. However, the underlying specific regulation mechanism should be further investigated. For example, the signaling pathways that control cell cycle and apoptosis needed to be further explored. Inflammatory processes have been suggested to play important roles in the pathogenesis of DR, and whether miR-346 regulates the expression of inflammatory markers in DR should be further investigated. Additionally, many ocular cells are affected in DR. For example, apoptosis of neural and vascular cells in the retina had been demonstrated to be a contributing mechanism of DR^[55]. This study indicated that miR-346 mediated the cell proliferation, apoptosis and oxidative stress in HG-induced damage of RPE cell. It was necessary to investigate whether miR-346 mediated these processes of other ocular cells. Only based on this, the roles of miR-346 could be extended to DR

in general. In conclusion, as per the current bioinformatic and experimental results, miR-346 is highly expressed in DR and plays an important role in HG-induced damage in human RPE cells. Therefore, miR-346 may play a key role in DR progression. However, in future, more rigorous, prospective, large sample sized experimental studies and long-term clinical trials are required to support this finding.

ACKNOWLEDGEMENTS

Foundations: Supported by the Social Development Project of Shaanxi Provincial Department of Science and Technology (No.2020SF-167); Supporting Fund Project of Shaanxi Provincial Department of Science and Technology Agency Project (No.2022SF-502); Xi'an Medical University 2022 Annual Scientific Research Capacity Improvement Plan Project (No.2022NLTS104).

Conflicts of Interest: Li P, None; Wang L, None; Liu Q, None; Du ZJ, None.

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