Int J Clin Exp Pathol 2016;9(2):1368-1375
www.ijcep.com /ISSN:1936-2625/IJCEP0018506

Original Article

Down-regulation of miR-34a promotes the cell proliferation and inhibits apoptosis in glaucoma

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Received October 26, 2015; Accepted January 21, 2016; Epub February 1, 2016; Published February 15, 2016

Abstract: Increasing evidence has reported the pivotal roles of miRNA in various diseases, including glaucoma. However, few have reported the roles of miR-34a in glaucoma progression. This study was aimed to investigate the potential effect of miR-34a expression on HTM and GTM cell proliferation and apoptosis and to explore the possible mechanism. The mRNA expression of miR-34a in HTM and GTM cells was detected using RT-PCR. The influences of miR-34a suppression on cell viability and apoptosis were analyzed using MTT and Annexin V-FITC assay respectively. Besides, effect of miR-34a on cell apoptosis-related protein expression was assessed using western blotting. Compared with the HTM cells, miR-34a was highly expressed in GTM cells. Subsequently, cell viability for both HTM and GTM cells was significantly increased by silencing miR-34a. The percentage of apoptotic GTM cells was significantly declined by silencing miR-34a. However, the HTM apoptosis cells were slightly but not significantly decreased by silencing miR-34a. Moreover, cell apoptosis-related protein including SIRT1 and Bcl-2 were significantly increased while Ac-p53 level was significantly decreased by silencing miR-34a. Taken together, our study suggested that miR-34a suppression may play pivotal roles in preventing HTM from transforming to GTM during glaucoma progression through regulating the cell proliferation and apoptosis. Our study may provide theoretical basis for the target therapeutic treatment of miR-34a in glaucoma.

Keywords: Glaucoma, miR-34a, cell apoptosis, cell proliferation, SIRT1 and Bcl-2

Introduction

Glaucoma is the second worldwide eye disease, which can lead to the irreversible blindness [1]. Types including the primary open angle glaucoma (POAG), primary angle-closure glaucoma (PACG) and exfoliation glaucoma (XFG) are the most common types for glaucoma [2]. Previous evidence has demonstrated that the pathogen mechanism for glaucoma is complicate, but it is has been widely accepted that glaucoma was affected by the mutual influence between genetic and environmental factors, thereinto, the genetic factors in glaucoma has become the hot spot for glaucoma pathogen research in recent years [3, 4]. The traditional clinical treatment method for glaucoma is intraocular pressure reduction, and combined with the drug or surgical therapy [5], but the treatment still remains unsatisfactory due to the side-effect brought by drug therapy or the complication in post-surgery. Hence, to investigate several useful targets for glaucoma treatment will be of great significance.

miRNAs are some endogenous, highly conserved non-coding RNAs 22-nt in length that function in a various biological processes at the transcriptional or post-transcriptional level by targeting the 3'UTR of genes [6]. Previous evidence has reported that a variety of miRNAs are involved in the underlying biology of glaucoma [7, 8]. For example, miR-34a or miR-20a suppresses cell proliferation and then prevents human trabecular meshwork (HTM) cells senescence [9], and the inflammatory marker of miR-146a and miR-493 suppress the cell senescence [9, 10]. The tumor suppressor role of miR-34a has been demonstrated in a variety of

Table 1. Primers used for targets amplification in this study

Name	Primer	Sequence (5'-3')
GAPDH	Sense	GGGTGGAGCCAAACGGGTC
	Antisense	GGAGTTGCTGTTGAAGTCGCA
SIRT1	Sense	CAGAGCAT CACACGCAAGC
	Antisense	CAGGAAACAG AAACCCCAGC
Bcl-2	Sense	TTGTGGCCTTCTTTGAGTTCGGTG
	Antisense	GGTGCCGGTTCAGGTACTCAGTCA

tumors such as colon cancer and prostate cancer via numerous mechanisms [11, 12]. However, few have reported the association of miR-34a with glaucoma progression.

In the current study, we investigated the expression of miR-34a on glaucoma cells using the HTM and glaucoma TM (GTM) cells and siRNA-mediated gene silencing. Various experimental methods were used to analyze the effects of miR-34a expression on HTM and GTM cell viability, apoptosis and apoptosis-related protein expression. This study was aimed to investigate the potential role of miR-34a in glaucoma pathogen and to explore the possible mechanism of its action.

Materials and methods

Cell culture and cell transfection

The adherent grown HTM and GTM cells were cultured in DMEM/F12 medium containing 20% fetal bovine serum (FBS; Hyclone, USA) in an atmosphere of 5% CO_2 at 37°C. Cells with the alignment of 70%-80% were cultured with D-hanks buffer (NaCl 8.01 g/L; KCl 0.4 g/L; $CaCl_2$ 0.14 g/L; $NaHCO_3$ 0.35 g/L; KH_2PO_4 0.06 g/L; Glucose 0.34 g/L) for the next generation production.

Cell proliferation assay

MTT (3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyltetrazolium bromide) assaywas used to assess the cell viability as previously described [13]. Briefly, after the cells were transfected with the siRNA-34a or siRNA control vectors for 36 h. Cells were adjusted to 5×10^3 cells for injection onto the 96-well plates. After 24 h of incubation, cells were centrifuged at 12,000 rpm, and then supernatant was removed. Followed by addition into 20 μL of MTT and then cultured for another 4 h. Finally, 150 μL of dimethylsulfoxide (DMSO) was used to mix with the cells for 10 min. Absorbance of cells in each well was observed at 570 nm under an absorption spectrophotometer (Olympus, Japan).

Cell apoptosis assay

The cell apoptosis was analyzed using the Annexin V-FITC cell apoptosis kit (Invitrogen, USA) according to manufacturer's protocol [14]. Briefly, after cells were transfected with the siRNA-34a and siRNA control vectors for 24 h, the medium was replaced with serum-free DMEM/F12 medium. Total cells were harvested and then washed using PBS buffer (PH 7.4) for 3 times, and then resuspended in the staining buffer. After that, 5 μL of annexin-V-FITC and 5 μL of propidium iodide (PI) were mixed with the cells. After being cultivated at room temperature for 10 min, mixtures were analyzed using the FACScan flow cytometry. Annexin V-positive and propidium iodide-negative cells were considered to be apoptotic cells.

Real time (RT)-PCR

Total RNA was extracted from the cells which were collected at 48 h using TRIzol Reagent (Invitrogen, CA, USA) as previously described [15] and was treated with RNse-free Dnase I (Promega Biotech, USA). Consequently, concentration and purity of isolated RNA were measured with SMA 400 UV-VIS (Merinton, Shanghai, China). Purified RNA at density of 0.5 μg/μL with nuclease-free water was used for cDNA synthesis with the PrimerScript 1st Strand cDNA Synthesis Kit (Invitrogen, USA). Expressions of targets in OVCAR-3 cells were detected in an Eppendorf Mastercycler (Brinkman Instruments, Westbury, NY) using the SYBR ExScript RT-PCR Kit (Takara, China). The total reaction system of 20 μL volume was as follows: 1 μL cDNA from the above PCR, 10 μL SYBR Premix EX Taq, 1 μL each of the primers (10 μM), and 7 μL ddH_2O. The PCR program was as follows: denaturation at 50°C for 2 min; 95°C for 10 min; followed by 45 cycles of 95°C for 10 s, and 60°C for 1 min. Melting curve analysis of amplification products was performed at the end of each PCR to confirm that only one product was amplified and detected. The GAPDH (Sigma, USA) was chosen as the internal control. Primers used for targets amplification were shown in **Table 1**.

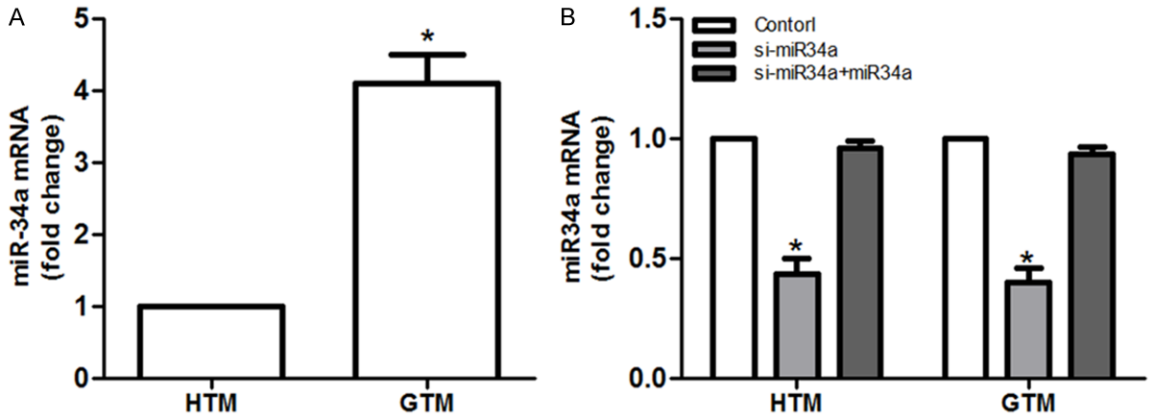

Figure 1. Expression of miR-34a in the healthy trabecular meshwork (HTM) and glaucoma trabecular meshwork (GTM) cells. A: Relative mRNA level of miR-34a was significantly increased in GTM compared to that in HTM cells, *: P<0.05 compared with the control group (HTM); B: The relative mRNA level of miR-34a was down-regulated by silencing miR-34a transfection in both HTM and GTM cells. *: P<0.05 compared with the control group (HTM).

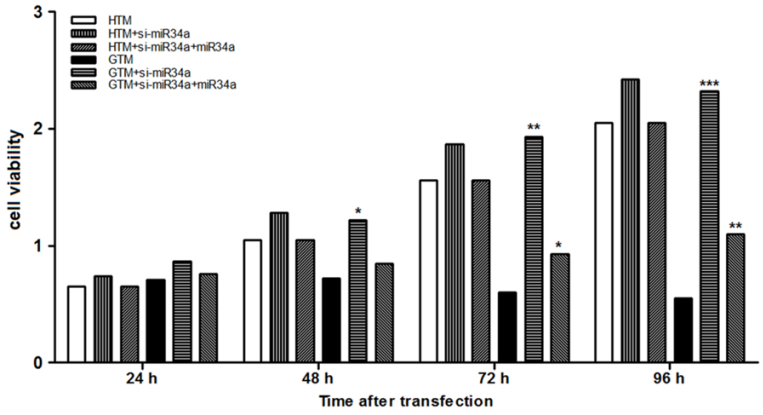

Figure 2. Effects of miR-34a suppression on HTM and GTM cell viability. The cell viability was slightly but not significantly increased by silencing miR-34a in HTM cells, and there was also no significant difference between the control group and the si-miR-34a + miR-34a group. Otherwise, when GTM cells were treated with siRNA-miR-34a, the cell viability was significantly increased at 48 h till 96 h. However, when cells were transfected with si-miR-34a + miR-34a, cell viability was decreased but was still significantly higher than that in control group. *: P<0.05, **: P<0.01, and ***: P<0.0001, compared with the control group (GTM).

Western blot analysis

Cells cultured for 48 h in each group were lapped with radioimmunoprecipitation (RIPA; Sangon Biotech, China) lysate containing phenylmethanesufonyl fluoride (PMSF; Sigma, USA), and then were centrifuged at 12,000 rpm for 10 min at 4°C. Supertanant was collected for the measurement of protein concentrations using BCA protein assay kit (Pierce, Rochford, IL). For western blotting [16], 40 μg of protein per cell lane was subjected to a 12% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transferred onto the polyvinylidencefluoride (PVDF) membrane (Mippore). Then the membranes were blocked in Tris-Buffered Saline Tween (TBST) containing 5% non-fat milk for 1 h at room temperature. Subsequently, the membranes were incubated with rabbit anti-human antibodies (SIRT1, Bcl-2, p53, Ac-p53, and GAPDH, 1:100 dilution, Invitrogen, USA) and overnight at 4°C. Then membrane was incubated with horseradish peroxidase-labeled goat anti-rat secondary antibody (1:1000 dilution) at room temperature for 1 h. Finally, the membranes were washed 3 times with 1× TBST buffer for 10 min each. The signals were detected after incubation with a chromogenic substrate using the enhanced chemiluminescence (ECL) method. Additionally, GAPDH served as the internal control. Additionally, GAPDH served as the internal control.

Statistical analysis

The total experiments were independently conducted 3 times. All the data were expressed as mean ± SD. The statistical analysis was calculated using Graph Prism 5.0 software (GraphPad Prism, San Diego, CA). The significant differ-

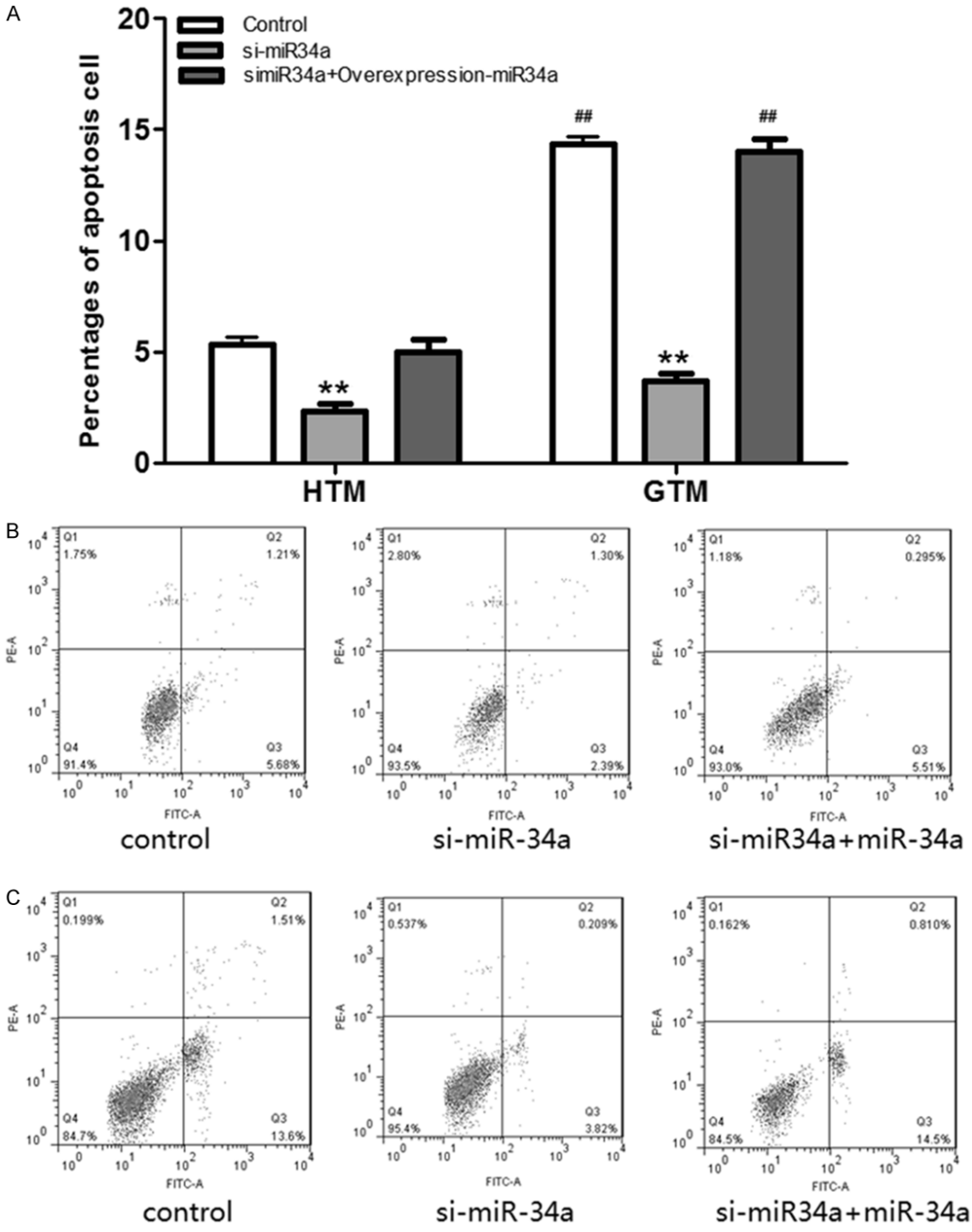

Figure 3. Effects of miR-34a suppression on HTM and GTM cells apoptosis. A: The percentage of apoptotic cells was decreased by silencing miR-34a in both HTM and GTM cells. Additionally, the percentage of apoptotic cells for GTM cells was higher than that in HTM cells. **: P<0.01 compared with the control group; and ##: P<0.01, compared with the control group (HTM). B, C: FITC-A assay showed the percentage of apoptotic cells in each group in HTM and GTM cells respectively.

ence between two groups was calculated using the one-way analysis of variance (ANOVA). The P<0.05 was considered as statistically significant.

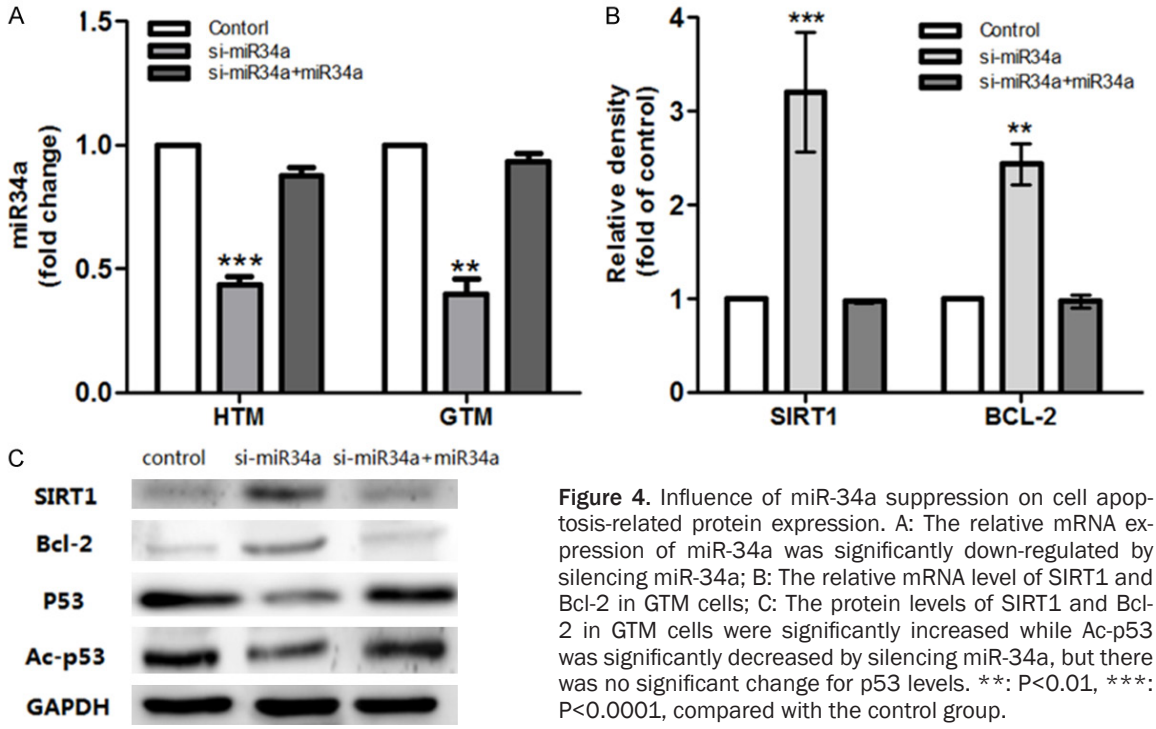

Figure 4. Influence of miR-34a suppression on cell apoptosis-related protein expression. A: The relative mRNA expression of miR-34a was significantly down-regulated by silencing miR-34a; B: The relative mRNA level of SIRT1 and Bcl-2 in GTM cells; C: The protein levels of SIRT1 and Bcl-2 in GTM cells were significantly increased while Ac-p53 was significantly decreased by silencing miR-34a, but there was no significant change for p53 levels. **: P<0.01, ***: P<0.0001, compared with the control group.

Results

mRNA expression of miR-34a in glaucoma cells

The results showed that the mRNA expression of miR-34a in GTM was significantly increased compared to that in HTM (P<0.05, **Figure 1A**), indicating that miR-34a may play certain roles in glaucoma pathogen. In addition, the mRNA level of miR-34a was significantly decreased by silencing miR-34a both in HTM and in GTM, but this effect was reversed when cells were transfected with both silencing miR-34a and miR-34a (P<0.05, **Figure 1B**).

miR-34a suppression inhibited glaucoma cell viability

The cell viability was slightly increased but not significantly increased in HTM by the silencing miR-34a with time increasing (**Figure 2**). However, the cell viability in GTM was significantly increased by silencing miR-34a at 48 h till 96 h (P<0.05), which was different to that in HTM at the same time point. Nevertheless, when cells were transfected with both siRNA-miR-34a and miR-34a, the cell viability in GTM was decreased but was also higher than that in control cells, suggesting that miR-34a suppression could inhibit glaucoma cell viability.

Effects of miR-34a expression on cell apoptosis

The cell viability assay showed that both HTM and GTM cell viability was partly blocked by silencing miR-34a, but not inhibited; therefore, we investigated the effect of miR-34a suppression on glaucoma cell apoptosis (**Figure 3**). The results showed that the percentage of apoptotic cells was significantly decreased by silencing miR-34a in HTM or GTM cells compared to the control (P<0.01, **Figure 3A**). Also, percentage of apoptotic cells in GTM (13.6%) was higher than that in HTM (5.68%). However, when cells were treated with siRNA-miR-34a and miR-34a, the percentage of apoptotic cells was returned as that in controls (**Figure 3B** and **3C**), indicating that silencing miR-34a could suppress cell apoptosis.

Effects of miR-34a expression on cell apoptosis-related protein expression

In order to investigate the potential mechanism for miR-34a suppression on glaucoma cell viability and apoptosis, we further analyzed the cell apoptosis-related proteins expression (**Figure 4**). The results showed that the relative mRNA level and protein expression of the apoptosis-related proteins including SIRT1 and Bcl-2 were significantly increased while acetylated-

p53 (Ac-p53) was significantly decreased in GTM cells compared with the control ($P<0.05$, **Figure 4B** and **4C**). Besides, the mRNA and protein level for p53 was slightly but not significantly decreased compared to the control. Additionally, when cells were treated with both siRNA-miR-34a and miR-34a, the levels of the total proteins including SIRT1, Bcl-2, P53 and Ac-p53 were almost the same as in the control.

Discussion

Increasing studies have reported the crucial roles on miRNAs in the biological in various diseases [7, 17], and miR-34a has been proved to be a tumor suppressor in a variety of tumors [11, 12]. To date, miR-34a has been reported to be involved in cell proliferation regulation in HTM senescence [17, 18]. In this study, we analyzed the potential effect of miR-34a suppression on GTM cell proliferation and apoptosis. Our data showed that miR-34a was highly expressed in GTM cells than that in HTM cells. Besides, the cell viability both for HTM and GTM cells was increased by silencing miR-34a. Moreover, GTM cell apoptosis except for HTM cells was inhibited by silencing miR-34a, and the cell apoptosis-related protein including SIRT1 and Bcl-2 were significantly increased while Ac-p53 was significantly decreased by silencing miR-34a.

It has been widely demonstrated miRNAs play pivotal roles in a variety of diseases biology such as glaucoma [8, 10, 18]. miR-34a was down-regulated in breast cancer and colon cancer [19]. Our results revealed that miR-34a was highly expressed in GTM cells compared to that in HTM cells (**Figure 1**), implying the correlation between miR-34a and glaucoma pathogen. Subsequently, we further analyzed the effects of miR-34a suppression on cell viability in HTM and GTM cells. The results showed that cell viability was significantly increased by silencing miR-34a both in HTM and GTM cells (**Figure 2**). Dutta et al proved that miR-34a overexpression on cell proliferation is cell type-dependent, namely, miR-34a suppression would increase the majority kinds of tumors [20]. The up-regulated miR-34a suppresses cell proliferation through regulating complicate signaling pathway in glioma [21, 22]. Besides, Smit-Mchride et al said that miR-34a expression is age-dependent increase in mouse eye [23]. Based on our results, we speculated that miR-34a suppression may be a suppressor for glaucoma progression via the involvement of cell proliferation.

Interestingly, the cell viability increased by silencing miR-34a in GTM cells was partly blocked by the co-transfection of siRNA-miR-34a and miR-34a vectors, we thereby investigated the effects of miR-34a suppression on cell apoptosis. For example, silencing miR-34a could inhibit chondrocyte apoptosis in rat osteoarthritis [24]. Also, miR-34a has been proved to be a tumor suppressor via regulating apoptosis in neuroblastoma or pancreatic cancer cells [25, 26]. In this study, the results revealed that the apoptotic GTM cells were decreased by silencing miR-34a (**Figure 3**), implying that miR-34a may be involved in the apoptosis of glaucoma.

Meanwhile, the cell apoptosis-related protein expression was analyzed by silencing miR-34a to investigate the potential mechanism. Sirtuin 1 (SIRT1) is an NAD-dependent deacetylasethat is involved in numerous biological processes including DNA-damage, apoptosis and proliferation [27]. It has been proved that miR-34a inhibits SIRT1 expression ultimately leads to apoptosis in colon cancer cells [28]. In this study, the expression levels of SIRT1 and Bcl-2 (the cell apoptosis suppressor) were significantly increased by silencing miR-34a (**Figure 4B**), suggesting that miR-34a suppression may be involved in suppressing cell apoptosis in glaucoma. On the other side, previous study proved that miR-34a is a p53-dependent tumor suppressor [29]. Besides, the deacetylase activities are necessary for normal cell proliferation and apoptosis, and the deacetylated p53 regulates cell growth and apoptosis [30]. Our results showed that the Ac-p53 was down-regulated by silencing miR-34a (**Figure 4C**), indicating that miR-34a suppression may inhibit cell apoptosis by down-regulating Ac-p53. Taken together, silencing miR-34a may inhibit GTM cell apoptosis through up-regulating the SIRT1 and Bcl-2 but down-regulating Ac-p53.

To sum up, the data presented in this study suggests that miR-34a suppression may play pivotal roles in glaucoma progression via regulating cell proliferation and apoptosis. Silencing miR-34a increases SIRT1 and Bcl-2 expression but down-regulates Ac-p53 in GTM. Our study

may provide theoretical basis for the possibility of target therapeutic treatment of miR-34a in glaucoma. However, further studies are still needed to explore the deep mechanism.

Acknowledgements

This research was supported by The College Students' Innovative Entrepreneurial Project (201510598023).

Disclosure of conflict of interest

None.

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