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Down-Regulation of MicroRNA-133b Suppresses Apoptosis of Lens Epithelial Cell by Up-Regulating BCL2L2 in Age-Related Cataracts

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

ABCDEFG Feng Zhang
BDE Weizhe Meng
BCD Bin Tong

Department of Ophthalmology, The People's Hospital of Beizhou, Beizhou, Shandong, P.R. China

Corresponding Author: Feng Zhang, e-mail: cataract133b@163.com
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Background: MicroRNA-133b (miR-133b) has been reported to be involved in many diseases, including ovarian cancer and osteosarcoma. Accumulating evidence suggests that miR-133b plays important roles in human disease. In this study, we aimed to investigate the molecular mechanism, including the potential regulator and signaling pathways, of BCL2L2.

Material/Methods: We first searched the online miRNA database (*www.mirdb.org*) using the "seed sequence" located within the 3'-UTR of the target gene, and then performed luciferase assay to test the regulatory relationship between miR-133b and BCL2L2. Western blot and real-time PCR were used to determine the expression of BCL2L2 in human samples or cells treated with miRNA mimics or inhibitors. Flow cytometry was conducted to evaluate the apoptosis status of the cells.

Results: We validated BCL2L2 to be the direct gene using a luciferase reporter assay. We also conducted real-time PCR and Western blot analyses to study the mRNA and protein expression level of BCL2L2 among different groups (control: n=29, cataract: n=33) or cells treated with scramble control, miR-133b mimics, BCL2L2 siRNA, and miR-133b inhibitors, and identified the negative regulatory relationship between miR-133b and BCL2L2. We also conducted experiments to investigate the influence of miR-133b and BCL2L2 on the viability and apoptosis of cells. The results showed that miR-133b positively interfered with the viability of cells, while BCL2L2 negatively interfered with the viability of cells, and that miR-133b inhibited apoptosis while BCL2L2 accelerated apoptosis.

Conclusions: BCL2L2 was the virtual target of miR-133b, and we found a negative regulatory relationship between miR-133b and BCL2L2. MiR-133b and BCL2L2 interfered with the viability and apoptosis of cells.

MeSH Keywords: Genes, bcl-2 • Apoptosis • MicroRNAs • Lenses

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Background

Age-related cataract is the most common cause of poor vision and blindness worldwide. The World Health Organization (WHO) reports that nearly 50% of blindness in the world (37 million blind people) is caused by cataracts [1]. Ultraviolet radiation exposure, smoking, aging, and various genetic factors have been established as risk factors for cataracts [2], but for alcohol consumption, antioxidants, and supplement use, the epidemiological evidence is still controversial [2]. Several studies have identified hyperglycemia and diabetes as risk factors for cataracts [3,4]. It has also been observed that obesity or central obesity [5–9] and serum lipid levels [9–11] can increase the risk of cataracts.

Recent studies on the apoptosis of lens epithelial cells (LECs) when exposed to toxic agents have demonstrated the importance of LECs in cataract formation [12]. It can be surmised from the literature [12] that cataract formation might be decreased by blocking apoptosis; it was shown in a recent study by Li et al. [12] that a large number of TUNEL-positive cells were detected in capsulotomy specimens from patients who underwent cataract surgery, but were not detected in normal specimens. It was also reported that a ladder in DNA was extracted from pooled capsulotomy samples, but was not found in the normal lens epithelia. It can be concluded from these data that apoptosis is a common cellular basis for non-congenital cataract formation [12].

MicroRNAs (miRNAs) are a class of small endogenous non-coding RNAs approximately 18–22 nucleotides (nt) in length, and miRNAs have been reported to be able to regulate the expression of genes post-transcriptionally by binding to the "seed sequence" within the 3'-untranslated regions (3'-UTR) of the mRNA of the target gene, thus repressing translation or accelerating mRNA degradation[13–16]. It has been shown in many studies in the past decade that abnormal expression of miRNAs is closely associated with the pathogenesis of many age-related diseases [17], such as cataract progression [18].

It has been shown that miR-133b is differentially expressed in the lens epithelial cells collected from age-related cataracts [19], and dysregulation of BCL2L2 has also been reported to be involved in the molecular mechanism of the apoptosis of lens epithelial cells [20,21]. By searching the online miRNA database, we found that BCL2L2 was a virtual target of miR-133b. In this study, we validated BCL2L2 as a target of miR-133b and verified the involvement of miR-133b and BCL2L2 in the development of age-related cataracts.

Material and Methods

Specimens

The present study enrolled age-related cataract patients (N=33, with no other eye diseases) who were admitted to the People's Hospital of Beizhou between September 2012 and August 2014. Fresh anterior lens capsules from each cataract patient were obtained during cataract surgery (phacoemulsification). Normal anterior lens capsule specimens (N=29, Eye Bank of the People's Hospital of Beizhou) were used as the control group. No cataract or any other eye disease was noted in the control group. The cataract group mean age was 65.45±8.65 years and included 16 males and 13 females. The control group mean age was 66.85±9.43 years and included 19 males and 14 females. This protocol of the study was approved by the Ethics Committee of the People's Hospital of Beizhou. All experiments were performed in accordance with the Declaration of Helsinki. Informed consent was obtained from all patients prior to the study.

Quantitative real-time polymerase chain reaction

microRNA expression levels were determined through qRT-qPCR. In brief, we extracted total RNA from sample tissues or cultured cells using an RNA extraction kit (Invitrogen, NY, USA) according to the manufacturer's guidance. cDNA was synthesized using random primer. RT-qPCR analysis for miR-133b was conducted using the ABI7500 (Applied Biosystems, USA) in accordance with the manufacturer's instructions. Primer sets are listed in Table I. Product specificity was confirmed using a melting curve analysis. The expression of RNA U6 small nuclear 2 (RNU6B) was also measured for internal control. All experiments were performed in triplicate with three separate samples. All results were presented as the expression of miR-133b relative to that of the internal control in BCL2L2 tissues and cell lines using the $2^{-\Delta\Delta CT}$method.

Cell culture and transfection

Lens epithelial cells (HLE-B3) were obtained from the Cancer Institute and Hospital, Chinese Academy of Medical Sciences (Beijing, China) and maintained in DMEM (Gibco, Carlsbad, USA) supplemented with 10% FBS (Hyclone, Logan, USA), 100 U/ml penicillin and 100 U/ml streptomycin under a 37°C humidified, 5% CO_2 environment. On transfection day, the cells were transfected with microRNA mimic, inhibitor, mimic scramble control, and inhibitor negative control (GenePharma, Shanghai, China) using Lipofectamine 2000 (Invitrogen, NY, USA) by following the manufacturer's instructions. After 48 h, the cells were harvested for the subsequent experiments.

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Apoptosis assay

Cell apoptosis rate assays by Annexin V-FITC/propidium iodide (PI) staining were conducted to assess the apoptosis rate of cells using flow cytometry (BD Biosciences, CA, USA). Briefly, after transfection, LPC cells ($1–5×10^5$) were collected and stained with Annexin V-FITC/PI using an apoptosis detection kit (Invitrogen, NY, USA). After 15 min of incubation at room temperature in the dark, the apoptotic cell percentages were measured through flow cytometry. The Annexin V immunofluorescence was presented on the X-axis and the plasma membrane integrity was presented on the Y-axis. Each experiment was conducted in triplicate.

Western blot

SDS-PAGE and Western blots were performed to determine the protein expression levels according to standard procedures. In brief, protein from cells was extracted using lysis buffer (2 mM Tris-Cl pH 7.5, 150 mM NaCl, 10% glycerol and 0.2% NP-40 plus a protease inhibitor cocktail). Protein levels of the cell lysate were quantified using a BCA protein assay kit (Beijing ComWin Biotech Co. Ltd., Beijing, China). The protein extracts were then loaded on 10% SDS-PAGE gel sand trans-blotted to PVDF membranes. After blocking in TBS-TB (TBS with 0.05% Tween-20, 0.05% BSA) containing 5% non-fat powdered milk for 1 h at 37°C, we incubated the membranes with primary antibody against BCL2L2 (Santa Cruz, CA, USA) or β-actin (Santa Cruz, CA, USA) at 4°C for 18 h. Subsequently the membranes were washed and incubated with secondary antibody at room temperature for 1 h. The antibodies used in this study are summarized in Table II. Finally, the protein blots were visualized using a Pierce ECL Western blotting kit (Thermo Fisher Scientific, Waltham, MA, USA). The abundance of protein bands was quantified using ImageJ (National Institutes of Health, Bethesda, Maryland, USA).

Luciferase activity assay

The 3'-UTR of the BCL2L2-containing miR-133b binding sites was amplified from human cDNA and subcloned into the pmiR-RB-REPORT™ vector (GenePharma, Shanghai, China). Meanwhile, a mutant 3'-UTR construct was also generated and introduced into the pmiR-RB-REPORT™ for a control vector. For reporter assays, LPCs were seeded into 24-well plates one day before transfection. Wild-type or mutant reporter plasmid, miR-133b mimic, and mimic control (GenePharma, Shanghai, China) were transfected to cells using Lipofectamine 2000 (Invitrogen, NY, USA). Luciferase activity was measured 2 days after transfection using a Dual-Luciferase Reporter Assay System in accordance with the manufacturer's guidance (Promega, Madison, WI, USA). To analyze the expression of BCL2L2 or miR-133b, the firefly luciferase activities were normalized to the Renilla luciferase activity. Each experiment was performed in triplicate with three separate samples.

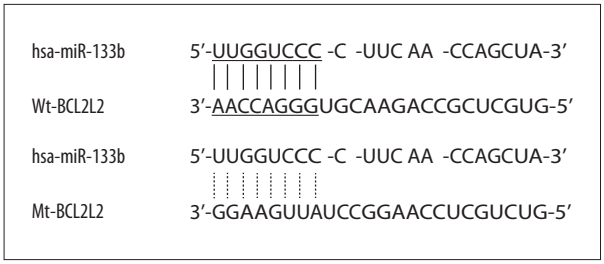

Figure 1. We used online miRNA target prediction tools to search the regulatory gene of miR-133b; BCL2L2 was the candidate target gene of miR-133b in cells with the 'seed sequence' in the 3'UTR.

Statistical analysis

All experiments were repeated three times at least. All quantitative data are expressed as the mean ± standard deviation (SD). Student-Newman-Keuls tests were performed and $p<0.05$ was considered statistically significant for all significant tests.

Results

BCL2L2 was virtual target of miR-133b

In order to understand the role of miR-133b in cataracts, we used online miRNA target prediction tools to search for the regulatory gene of miR-133b, and consequently identified BCL2L2 as the candidate target gene of miR-133b in VSMC cells with the "seed sequence" in the 3'UTR (Figure 1). Furthermore, to validate the regulatory relationship between miR-133b and BCL2L2, we also conducted luciferase activity reporter assays in cells. We observed that the luciferase activity from the cells co-transfected with miR-133b and wild-type BCL2L2 3'UTR decreased significantly (Figure 2), while cells co-transfected with miR-133b and mutant-type BCL2L2 3'UTR were comparable to the scramble control (Figure 2). The results confirmed that BCL2L2 was a validated target of miR-133b in cells. To further explore the modulatory relationship between miR-133b and BCL2L2, we then analyzed the correlation between the expression levels of miR-133b and BCL2L2 mRNA in the tissues (n=62); they showed a negative regulatory relationship (Figure 3).

Determination of expression patterns of miR-133b and BCL2L2 in tissues with different groups

The tissues of two different groups (control: n=29, cataract: n=33) were used to further explore the impacts on the interaction between miR-133b and BCL2L2 3'UTR. Using real-time PCR,

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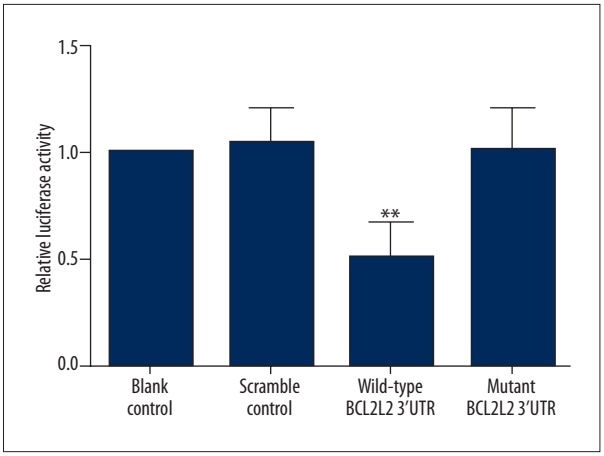

Figure 2. Luciferase activity reporter assay was conducted to verify BCL2L2 as the direct target gene of miR-133b (** denotes P<0.01).

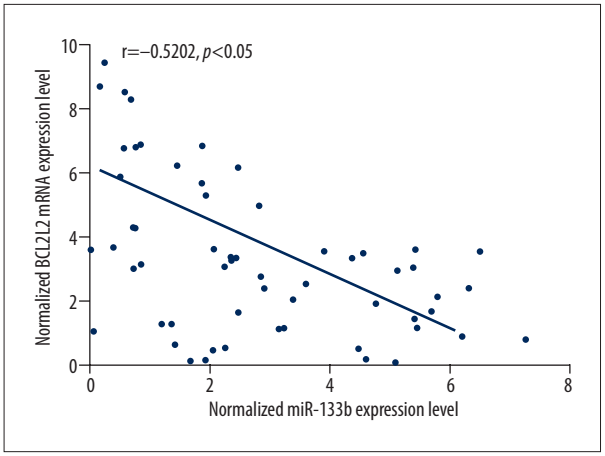

Figure 3. To further explore the effects on the interaction between miR-133b and BCL2L2, we used real-time PCR to detective the expression of the mRNA of miR-133b and BCL2L2, and BCL2L2 protein was measured by densitometry analysis. The results showed a negative regulatory relationship between miR-133b and BCL2L2 between cataract and control (cataract =33, control, n=29).

we found that the expression of miR-133b decreased in cataract groups (Figure 4A) compared with the control group, while the expression of BCL2L2 mRNA (Figure 4B) increased in the cataract group compared with the control group; the expression of BCL2L2 protein (Figure 4C) was measured by densitometry analysis, and was found to increase in the cataract group compared with the control group. To further validate the hypothesis of the negative regulatory relationship between miR-133b and BCL2L2, we investigated the mRNA/protein expression level of BCL2L2 of lens epithelial cells, by transfecting the cells with scramble control, miR-133b mimics, BCL2L2 siRNA and miR-133b inhibitors. As shown in Figure 5, the BCL2L2 protein (upper panel) and mRNA expression levels (lower panel) of cells treated with miR-133b mimics and BCL2L2 siRNA were apparently lower than the scramble control, while that of cells treated with miR-133b inhibitors was apparently higher than the scramble control, validating the negative regulatory relationship between miR-133b and BCL2L2.

miR-133b and BCL2L2 interfered with the viability of cells

We also investigated the relative viability of cells when transfected with scramble control, miR-133b mimics, BCL2L2 siRNA and miR-133b inhibitors. Cells transfected with miR-133b inhibitors showed evidently down-regulated viability (Figure 6A) when compared with the scramble controls, while cells transfected with miR-133b mimics and BCL2L2 siRNA showed comparably higher viability, indicating that miR-133b positively interfered with the viability of cells, while BCL2L2 negatively interfered with the viability of cells.

miR-133b and BCL2L2 interfered with the apoptosis of cells

We then investigated the relative apoptosis of cells when transfected with scramble control, miR-133b mimics, BCL2L2 siRNA and miR-133b inhibitors. When transfected with miR-133b mimics and BCL2L2 siRNA, the number of surviving cells was greater and the number of apoptotic cells was less than in the scramble controls, while cells transfected with miR-133b inhibitors showed comparably fewer surviving cells and more apoptotic cells. These results indicated that miR-133b inhibited apoptosis while BCL2L2 accelerated apoptosis.

Discussion

Age-related cataracts are divided into three major types: nuclear cataracts, posterior subcapsular cataracts and cortical cataracts. Regardless of type, all cataracts are linked to apoptotic cell death or abnormal growth in the lens. Numerous studies of the mechanisms of cataract pathogenesis have found that dysregulated apoptosis of LECs plays an important role in cataract formation. For cultured LECs, exposure to UV light or subjection to calcium iontophoresis or H_2O_2 treatment can increase the expression of the oncogenes *c-Myc* and *c-Fos* and pro-apoptotic protein caspase-3, and thus induce cell death[22]. In a study of human epithelial cells by Peng et al. [23], quantitative fluorescent PCR was used to detect the expression of the miRNAs let-7a, let-7b, and let-7c in cataract patients, and it was found that let-7b expression levels were positively correlated with age, while the expression of let-7a and let-7c was not linked to lens opacity or age. It was also found that let-7b suppressed the expression of *bcl-2* and thus promoted cell death. In addition, it was concluded from the literature[24] on microarray analysis that in normal human lens epithelial cells miR-923 and let-7b were not expressed, whereas in the

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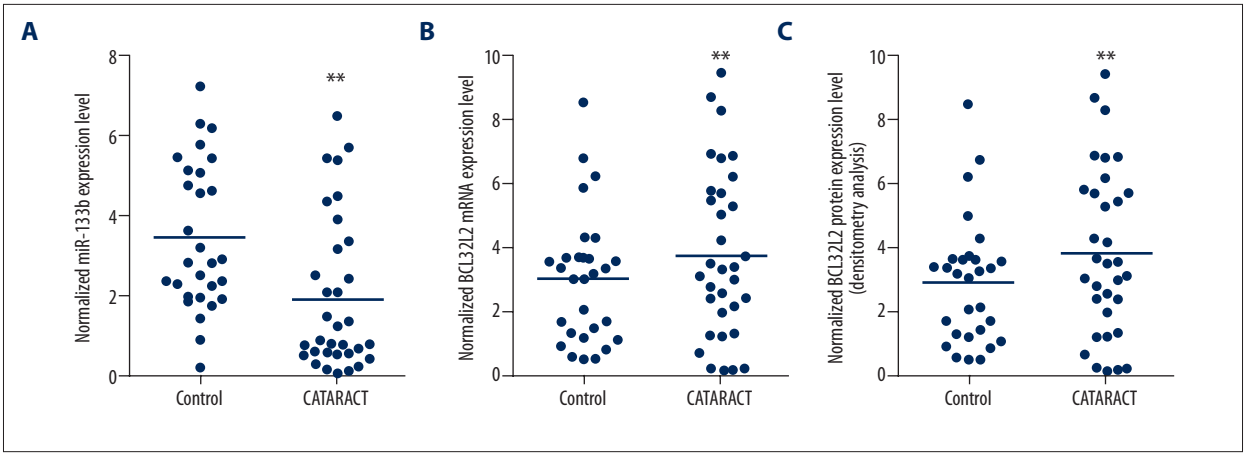

Figure 4. The expression of miR-133b decreased in the recurrence group (**A**) compared with normal control group while the expression of BCL2L2 mRNA (**B**)and protein (**C**) increased in cataract group compared with control group (** denotes P<0.01).

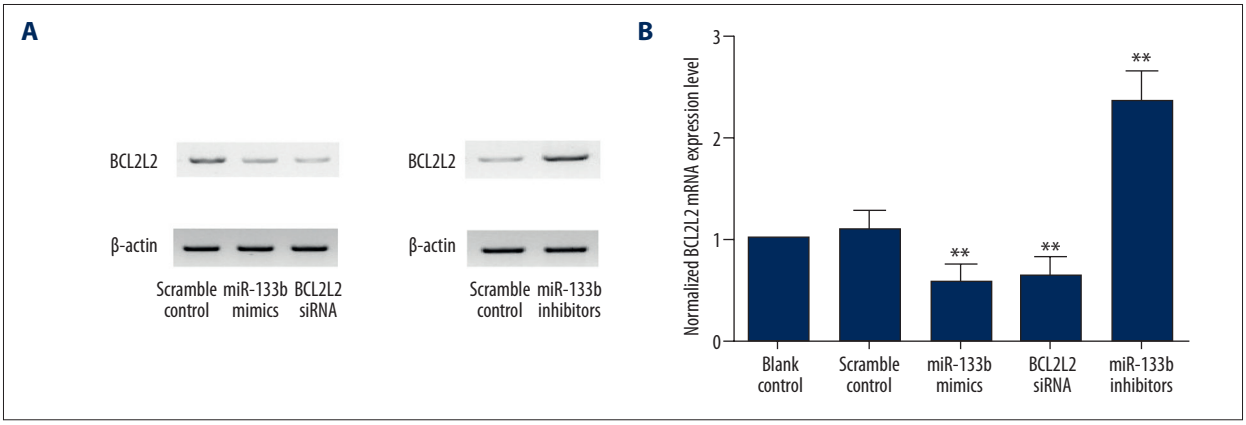

Figure 5. When transfected with the prostate cancer stem cells with scramble control, miR-133b mimics, BCL2L2 siRNA, and miR-133b inhibitors, the expression level of BCL2L2 protein (upper panel) and mRNA (lower panel) treated with miR-133b mimics and BCL2L2 siRNA decreased, while cells treated miR-133b inhibitors increased (** denotes P<0.01).

corresponding cells from cataract patients their expression was increased. In studies on animals, it was found that many miRNAs, such as let-7b, miR-31, miR-204, miR-26a, miR-184, and miR 125b, were expressed in the mouse lens[25].

Patron et al. (2012) demonstrated that miR-133b takes both anti-apoptotic and pro-apoptotic proteins as its target and controls the apoptotic process in various cell types. It was also revealed that transfection with miR-133b made the Hela cells sensitive to the death of tumour necrosis factor-alpha (TNF-α) [26]. The measurement of hERG activity, which promotes apoptosis, demonstrates the cell type- and environment-specific influences on apoptosis. For example, the apoptosis of HL-1 cells and prostatic cancer cells[27], as well as that of ovarian cancer cells [28], was accelerated by selectively blocking the hERG channel. This evidence suggests that miR-133b might increase arsenic-induced apoptosis in U251 glioma cells by targeting the hERG. In this study, we used online miRNA target prediction tools to search for the regulatory gene of miR-133b, and consequently identified BCL2L2 as the candidate target gene of miR-133b. Furthermore, we also conducted luciferase activity reporter assays in cells: we observed that the luciferase activity of the cells co-transfected with miR-133b and wild-type BCL2L2 3'UTR decreased significantly (Figure 2), while cells co-transfected with miR-133b and mutant-type BCL2L2 3'UTR were comparable to the scramble control. In addition, we analyzed the correlation between the expression levels of miR-133b and BCL2L2 mRNA in the tissues (n=62): they showed a negative regulatory relationship (Figure 3).

Yin et al. (1994) demonstrated that the proteins of the Bcl-2 family are crucial for the intrinsic apoptotic pathway. When apoptosis occurs, cytochrome c is released by the mitochondria into the cytosol, thus inducing the formation of the Apaf-1: caspase-9 holoenzyme. Bcl-2 can maintain the integrity of the mitochondrial membrane and thus prevent the release of cytochrome c. As a member of the Bcl-2 family, Bcl-W is the regulator of apoptosis, and is also known as BCL2L2. Bae et al. (2006) [29]

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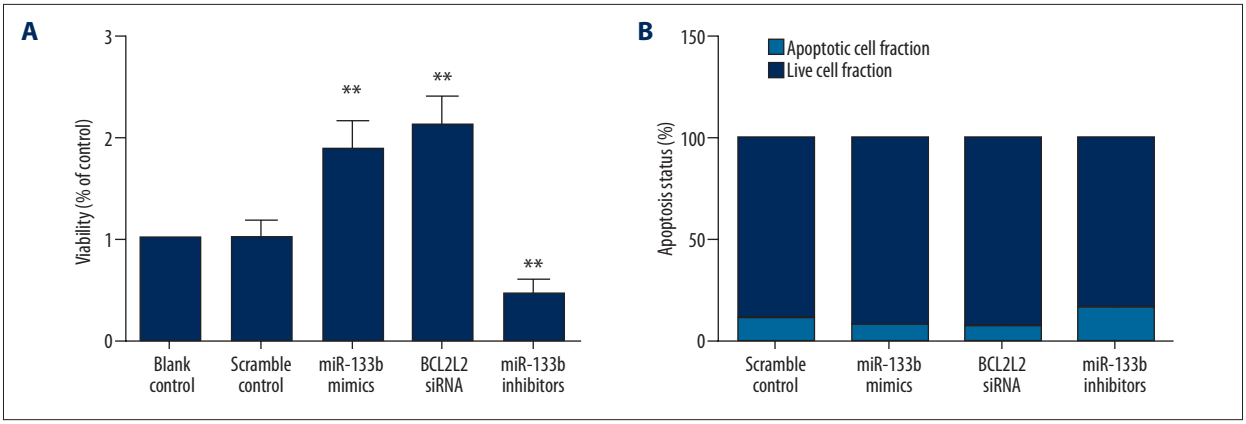

Figure 6. Cells transfected with miR-133b inhibitors showed evidently downregulated viability (**A**), while cells transfected with miR-133b mimics and BCL2L2 siRNA showed comparably higher viability. Cells transfected with miR-133b mimics and BCL2L2 siRNA inhibited apoptosis while cells transfected with miR-124 inhibitors accelerated apoptosis (**B**) (** denotes P<0.01).

showed that BCL2L2 is similar to its close relative Bcl-2, and is involved in the progression and maintenance of tumors. Gibson et al. (1996) [30] demonstrated that under cytotoxic conditions BCL2L2 suppresses the apoptosis and improves the survival of cells. Brady and Gil-Gomez (1998) [31] showed that the BAX and Bcl-2 families are major proteins associated with apoptosis. If BAX is overexpressed and then transported to the organelle, the mitochondrial apoptotic pathway is available. In contrast, BAX is bound to and inhibited by Bcl-2 proteins. LaCasse et al. (2008) [32] and Adams and Cory (2007) [33] demonstrated that in numerous cancer cell types the Bcl-2 family is overexpressed, which offers a therapeutic target for anticancer drugs that are designed to block these proteins and thus promote apoptosis. In this study, we collected the tissues of two different groups (control: n=29, cataract: n=33), and found that the expression of miR-133b decreased in cataract groups (Figure 4A) compared with the control group, while the expression of BCL2L2 mRNA (Figure 4B) increased in the cataract group compared with the control group. By further *in vitro* analysis, we showed that cells transfected with miR-133b inhibitors showed evidently down-regulated viability (Figure 6A) when compared with the scramble controls, while cells transfected with miR-133b mimics and BCL2L2 siRNA showed comparably higher viability, indicating that miR-133b positively interfered with the viability of cells, while BCL2L2 negatively interfered with the viability of cells. Subsequently, we performed apoptosis analysis and showed that when transfected with miR-133b mimics and BCL2L2 siRNA, the number of surviving cells was greater and the number of apoptotic cells was less than in the scramble controls, while cells transfected with miR-133b inhibitors showed comparably fewer surviving cells and more apoptotic cells. These results indicated that miR-133b inhibited apoptosis while BCL2L2 accelerated apoptosis.

Conclusions

We demonstrated that BCL2L2 was a validated target of miR-133b, and that there existed a negative regulatory relationship between miR-133b and BCL2L2. MiR-133b and BCL2L2 interfered with the viability and apoptosis of cells, which may contribute to the development of cataracts.

Conflict of interest

None.

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