Biochimica et Biophysica Acta 1842 (2014) 2439–2447

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

MicroRNA-125b inhibits lens epithelial cell apoptosis by targeting p53 in age-related cataract

Yu Qin [a], Jiangyue Zhao [a], Xiaojie Min [a], Mingwu Wang [b], Wenting Luo [c], Di Wu [a], Qichang Yan [a], Jing Li [a], Xinwei Wu [a], Jinsong Zhang [a,*]

[a] Department of Ophthalmology, the Fourth Affiliated Hospital of China Medical University, Eye Hospital of China Medical University, Key Lens Research Laboratory of Liaoning Province, China
[b] Department of Ophthalmology and Vision Science, the University of Arizona College of Medicine, Tucson, AZ, USA
[c] Key Laboratory of Health Ministry for Congenital Malformation, Shengjing Hospital, China Medical University, Liaoning, China

ARTICLE INFO

Article history:
Received 30 June 2014
Received in revised form 9 September 2014
Accepted 4 October 2014
Available online 13 October 2014

Keywords:
microRNA-125b
p53
Apoptosis
Gene expression regulation
Cataract

ABSTRACT

MicroRNA-125b (miR-125b) has been implicated in a variety of diseases as either repressors or promoters, and plays crucial roles in many cellular processes such as cell differentiation, proliferation and apoptosis. Age-related cataract has become one of the most serious problems facing the aging population in the world. The purpose of this study was to investigate the role of miR-125b in the development of age-related cataract. We demonstrated that miR-125b was downregulated in both age-related cataract tissue and lens epithelial cell apoptosis induced by UV irradiation. We also identified the impact of miR-125b on apoptosis in a lens epithelial cell line. *In vitro*, miR-125b regulates human lens epithelial cell apoptosis at least in part by directly targeting p53. In addition, an inverse relationship between miR-125b and p53 expression was seen in age-related cataract tissue. In conclusion, this study suggests that miR-125b might be closely involved in the pathogenesis of cataract, and has the potential to be a diagnostic biomarker or even a therapeutic modality for cataract.

1. Introduction

With the advancement in economy, health and education, population aging is becoming increasingly societal burden to mankind. Cataract, one of the common age-related diseases, remains the leading cause of blindness worldwide [1]. It has been reported that age-related cataracts are responsible for nearly half of all blindness worldwide and half of all visual impairment in the USA [2–4]. A recent Italian epidemiological study has shown that 96% of people aged over 60 can be found with different severities or forms of lens opacity [5]. It is estimated by WHO that there will be 40 million people worldwide with cataract blindness by 2025 [6]. Age-related cataract results in severe visual impairment, lowers quality of life, and has a significant impact on health resources and social economy.

Challenges remain in fighting cataract across the world, including large number of cataract patients, uneven distribution of medical resources, cost of operation, and development of posterior capsular opacification after surgery. As a result, although operation is currently the most effective cataract therapy, potential benefits warrant exploring nonoperative treatment of cataract. A poor understanding of the pathogenesis of cataract has been a hurdle in such a pursuit. Previous evidence indicated that lens epithelial cell apoptosis appears to be a common cellular basis for non-congenital cataract development in human and animals [7]. Therefore, study of lens epithelial cell apoptosis may shed new light in understanding the mechanism of cataract development and progression. Knowledge gained about microRNA (miRNA) has provided new opportunities in studying lens epithelial cell apoptosis.

MiRNAs are a group of endogenous small non-coding RNAs, approximately 20 to 25 nucleotides in length, which regulate gene expression posttranscriptionally [8,9]. MiRNAs regulate mRNA degradation or translational interference by binding to a complementary sequence found in the 3′-untranslated regions (UTR) of target gene mRNAs [10]. In the past decade, more and more studies have demonstrated that aberrant expression of miRNAs was tightly linked to the pathogenesis of many age-related disease [11–14], including cataract progression [15,16]. MiRNA research on the treatment of diseases has also made new breakthroughs [17–19].

MicroRNA-125b (miR-125b), a highly conserved miRNA throughout diverse species from nematode to human, has been implicated in a variety of diseases as either repressors or promoters [20]. MiR-125b

Abbreviations: miR-125b, microRNA-125b; miRNA, microRNA; UTR, untranslated region; UV, ultraviolet; PBS, phosphate buffer saline; RT-qPCR, real-time quantitative polymerase chain reaction; RNU6B, RNA U6 small nuclear 2; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; siRNA, small interfering RNA; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

* Corresponding author at: Department of Ophthalmology, The Fourth Affiliated Hospital of China Medical University, Eye Hospital of China Medical University, Key Lens Research Laboratory of Liaoning Province, China. No. 11 Xinhua Road, Heping District, Shenyang City, Liaoning Province 110005, China. Tel: +86 13940123417; fax: +86 24 62689311.
E-mail addresses: qinyubb@126.com (Y. Qin), zhaojiangyue@hotmail.com (J. Zhao), minxiaojie789@126.com (X. Min), mingwuwang@hotmail.com (M. Wang), lvirginia@163.com (W. Luo), wudiq7@126.com (D. Wu), cmu4h-yqc@126.com (Q. Yan), jingliwater123@163.com (J. Li), happy.wei@163.com (X. Wu), zhangjs0331@126.com (J. Zhang).

http://dx.doi.org/10.1016/j.bbadis.2014.10.002
0925-4439/

plays crucial roles in cellular processes such as cell differentiation, proliferation and apoptosis by targeting many different transcription factors [21], matrix-metalloprotease [22,23], and growth factors [24].

However, little is known about the role of miR-125b in the progression of lens disease, including cataract. This study found, for the first time, the crucial role of miR-125b and its direct target gene p53 in age-related cataract. We demonstrated that miR-125b was downregulated in age-related cataract tissue. We further observed that miR-125b was inversely related to the level of p53 in age-related cataract tissue. Additionally, we showed that miR-125b regulated human lens epithelial cell apoptosis at least in part by directly targeting p53.

2. Materials and methods

2.1. Specimens

Fresh specimens of anterior lens capsule of age-related cataract were obtained from cataract patients with no other eye diseases during phacoemulsification at the Fourth Affiliated Hospital of China Medical University between September 2012 and August 2014. Normal anterior lens capsule specimens were obtained from the Eye Bank of the Fourth Affiliated Hospital of China Medical University. This study was approved by the hospital's Ethic Committee and conducted with the approval of the institutional review board at the Fourth Affiliated Hospital of China Medical University. Written informed consent was obtained from all patients for all clinical investigation conducted.

2.2. Ultraviolet (UV) irradiation of cultured cells

Twin 15 W UVB lamps with a filter assembly (XX-15B, Spectroline, Westbury, NY, USA) were used for irradiation; these lamps emit an energy spectrum of 280–320 nm with a high fluence in the UVB region and a maximum emission peak at 312 nm. The intensity and dose were measured using a UVX Radiometer connected to a UVX-31 Sensor (calibration wavelength 312 nm) (both were from UVP Inc., San Gabriel, CA, USA). Before UV irradiation, cells (1×10^5/well) were washed with Dulbecco's phosphate buffer saline (PBS) and then changed to 0.5 ml PBS in each well. Human lens epithelial cells were irradiated without a plastic dish lid with an intensity of 360 μW/cm^2. Time of irradiation was varied at 5 min, 10 min, 15 min, 20 min, 25 min and 30 min. After exposure, the PBS was immediately replaced by fresh medium for a continued incubation of another 4 h.

2.3. Real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA from fresh tissues and cell lines was extracted using Trizol® Reagent (Invitrogen, NY, USA) following the manufacturer's instructions. The quality of RNA was examined using an UV–Vis spectrophotometer UV-1800 (Shimadzu, Japan). RNA integrity was verified using 1.5% agarose gel electrophoresis with OD260/280 between 1.8 and 2.0, and RNA 28 s/18 s > 1.

RT-qPCR analysis of miR-125b was carried out using RT primer and TaqMan probe of miR-125b (Ribobio, Guangzhou, China) on an ABI 7500 (Applied Biosystems, USA) according to the manufacturer's protocol. Expression of RNA U6 small nuclear 2 (RNU6B) was used as an endogenous reference control. Each RT-qPCR analysis was repeated twice, using three independent specimens. The relative abundance of miR-125b in tissues and cell lines was calculated using the equation $RQ = 2^{-\Delta\Delta CT}$.

For RT-qPCR analysis of p53, RNA was reverse-transcribed with PrimerScript RT reagent kit (Takara, Dalian, China). The resulting cDNA was amplified using p53 primers with SYBR Premix Ex TaqTM II (Takara, Dalian, China) with the following parameters 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s, and at 60 °C for 30 s. Primers for p53 were as follows: forward 5′-CAGCAGTCAAGCACTGCCAAG-3′, reverse 5′-AGACAGGCATGGCACGGATAA-3′. Primers for endogenous β-actin were: forward 5′-CATCCGTAAAGACCTCTATGCCAAC-3′, reverse 5′-ATGGAGCCACCGATCCACA-3′. In order to ensure product specificity, melting curve analysis was performed at the end of the cycles. The relative quantity of p53, normalized to β-actin, was calculated based on the equation $RQ = 2^{-\Delta\Delta CT}$.

2.4. Cell culture and transfection

SV40 T-antigen-transformed human lens epithelial cell line (SRA01/04 cell) [25] was gifted from Dr. Yi-sin Liu, Doheny Eye Institute, US. SRA01/04 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco® Invitrogen, Carlsbad, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, USA), 100 U/ml penicillin, and 100 U/ml streptomycin. Cells were incubated in a humidified 37 °C incubator containing 5% CO_2.

SRA01/04 cells were transfected using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's protocol. MiR-125b mimic, mimic negative control, inhibitor and inhibitor negative control were from RiboBio (Guangzhou, China) and transfected into SRA01/04 cells. The complexes containing the mimic or mimic negative control were added to cells at a final concentration of 50 nM, while the mixture containing the inhibitor or inhibitor negative control was at a final concentration of 100 nM. Moreover, small interfering RNA (siRNA) for p53 and a negative control were designed and synthesized by GenePharma (Shanghai, China) and transfected into SRA01/04 cells. The sequences of the p53 siRNA were 5′-CUACUUCCUGAAAACAACGTT-3′ (sense), and 5′-CGUUGUUUUCAG GAAGUAGTT-3′ (antisense). After 48 h, the expression of miR-125b was detected by RT-qPCR, the expression of p53 was tested by RT-qPCR and Western blot, and the cell apoptosis rate was measured by flow cytometry.

2.5. Terminal deoxynucleotidyl transferase deoxy-UTP-nick end labeling assay (TUNEL assay)

All the anterior lens capsules with adherent lens epithelium cells were placed in tubes filled with fortified balanced salt solution after being removed out of the eyes. Then anterior capsules were transferred into 10% neutral-buffered formalin immediately. After fixation, 15 capsules of each group were further dehydrated through graded alcohols, cleared in xylene, and embedded in paraffin. And then 4 μm sections were cut for TUNEL fluorescein analysis.

Sections were rehydrated through graded alcohols and the terminal deoxynucleotidyl transferase deoxy-UTP-nick end labeling (TUNEL) assay was performed using a standard kit, fluorescein DeadEnd™ Fluorometric TUNEL System (Promega, Madison, WI). The TUNEL assay detects the ends of DNA fragments formed during the apoptosis

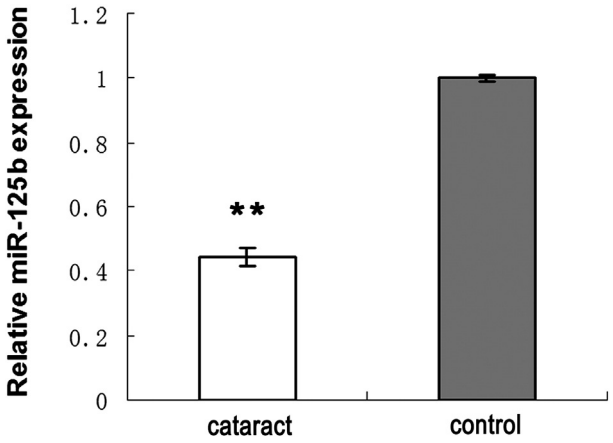

Fig. 1. MiR-125b was downregulated in age-related cataract lens tissue. Downregulated expression of miR-125b was demonstrated in 56 fresh specimens of anterior lens capsule of age-related cataract using RT-qPCR ($n = 3$, mean ± SD, $^{**}P < 0.001$).

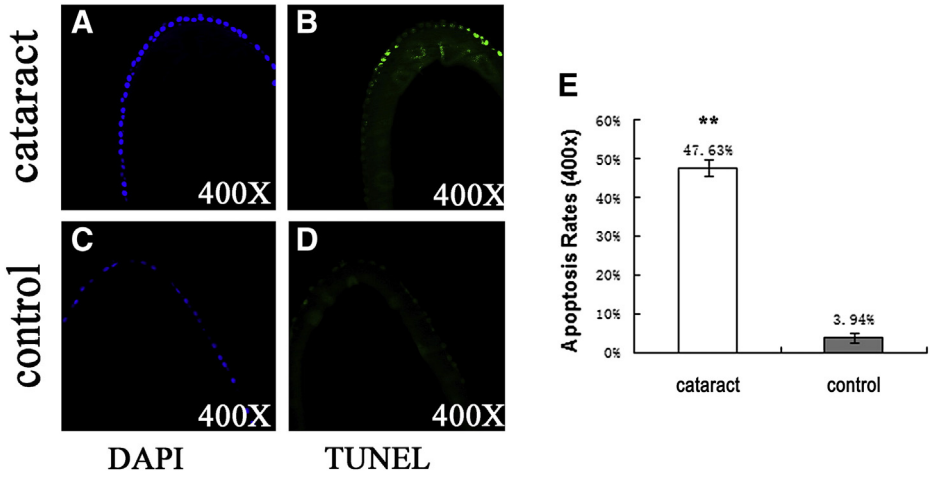

Fig. 2. Apoptosis analysis by TUNEL staining. (A)&(B) TUNEL positive cells were observed in large amounts in cataract group. (C)&(D) TUNEL positive cells were rarely found in normal lens group. (E) The higher cell apoptosis rate was detected in cataract group than normal lens group ($n = 60$, mean ± SD, $^{**}P < 0.001$).

process. To differentiate between nonspecific cells, counterstaining was performed using 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) in both groups. Then, all tissue sections were covered with a mounting medium. Bright-field and fluorescence images of sections were taken using Olympus microscope (BX51, Olympus) with a SPOT camera.

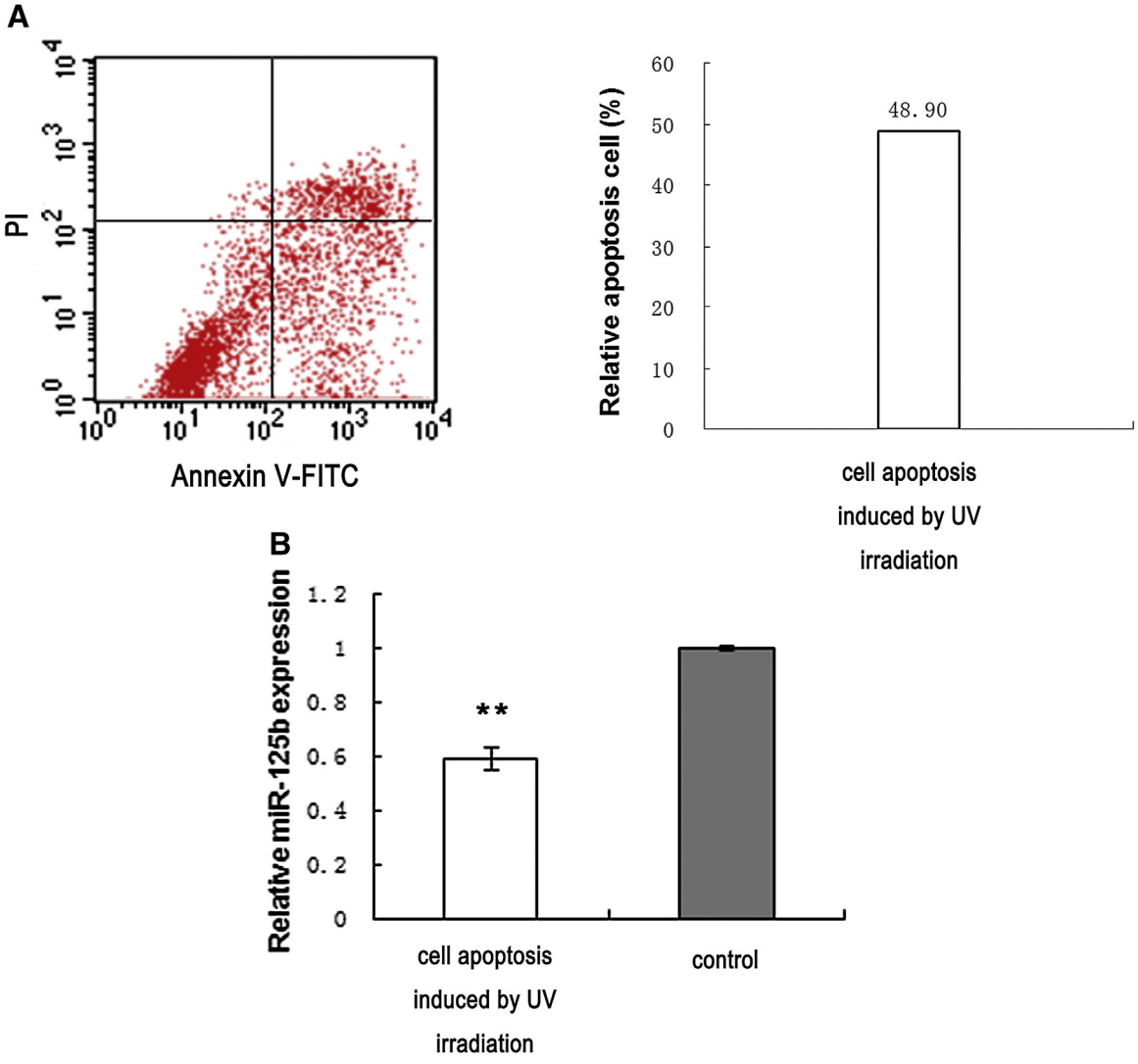

Fig. 3. (A) Lens epithelial cell apoptosis induced by UVB irradiation in SRA01/04 cell line. Cells were irradiated with an intensity of 360 μW/cm^2 for 25 min to achieve an effective dosage of 540 mJ/cm^2. Cell apoptosis rate was approximately 48.90% under this condition. (B) The expression of miR-125b as detected by RT-qPCR was downregulated during lens epithelial cell apoptosis ($n = 3$, mean ± SD, $^{**}P < 0.001$).

2.6. Cell apoptosis rate assayed by flow cytometry

After UV irradiation or 48 h after transfection, SRA01/04 cells ($1–5 \times 10^5$/well) were collected and then subjected to an apoptosis assay. Apoptosis was determined by annexin V/propidium iodide staining with the apoptosis detection kit (KenGEN, China). After incubation for 15 min at room temperature in darkness, the specimens were assessed by flow cytometry (BD Biosciences, CA, USA). Plasma membrane integrity was shown on the Y-axis and annexin V immunofluorescence was shown on the X-axis. Assays were performed in three independent experiments.

2.7. Western blot

SDS-PAGE and Western blots were performed according to standard procedures. Cells were extracted with lysis buffer containing 150 mM NaCl, 1% NP-40, 0.1% SDS, 2 mg/ml aprotinin and 1 mM PMSF for 30 min at 4 °C. Protein extracts were separated on 10% SDS-PAGE gels and transferred onto PVDF membranes. After blocking in TBST containing 25 mmol/l Tris–HCl, pH 7.5, 137 mmol/l NaCl, 2.7 mmol/l KCl and 0.05% Tween20 with 5% nonfat milk for 1 h at 37 °C, membranes were incubated with primary antibody against p53 (Santa Cruz, CA, USA) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz, CA, USA) in TBST with 5% nonfat milk at 4 °C overnight. Membranes were extensively washed three times with TBST and incubated with secondary antibody at room temperature for 1 h. After additional washes with TBST, proteins were visualized with an ECL kit (Santa Cruz, CA, USA). The intensity of protein bands was quantified using Image J software (National institutes of Health, Md, USA).

2.8. Luciferase activity assay

The 3′-UTR of the p53 gene including conserved binding sites for miR-125b was amplified from human cDNA. The amplified fragments were inserted into the pmiR-RB-REPORT™ vector (Ribobio, Guangzhou, China). A mutant 3′-UTR fragment, with mutations in seed binding sites, was generated. Similarly, the fragment of p53-3′-UTR mutant was inserted into the pmiR-RB-REPORT™ control vector at the same sites. For reporter assays, SRA01/04 cells were seeded into 24-well plates, cultured overnight, then cotransfected with wild-type (mutant) reporter plasmid, miR-125b mimic, and mimic control (Ribobio, Guangzhou, China) using Lipofectamine 2000 (Invitrogen, USA). Luciferase activity was analyzed 48 h post-transfection using Dual-Luciferase Reporter Assay System according to the manufacturer's instructions (Promega, Madison, WI, USA). The Renilla luciferase plasmid was used as an endogenous control. Each experiment was repeated twice, using three independent specimens.

2.9. Statistical analysis

All experiments were repeated twice at least. All data were reported as mean ± standard deviation (SD). Differences were assessed by an independent sample *t*-test. $P < 0.05$ was considered statistically significant. SPSS16.0 statistical software was used for statistical analysis.

3. Results

3.1. MiR-125b was downregulated in anterior lens capsule of age-related cataract

A total of 56 fresh specimens of anterior lens capsule of age-related cataract were examined for the expression of miR-125b and compared with an equal number of normal anterior lens capsule specimens. Compared with controls, the expression level of miR-125b as detected by RT-qPCR was significantly lower in the anterior lens capsules of age-related cataract (Fig. 1).

3.2. TUNEL positive cell count was higher in anterior lens capsule of age-related cataract

Apoptotic cells of 15 fresh specimens of anterior lens capsule of age-related cataract were detected by TUNEL assay and compared with an equal number of normal anterior lens capsule specimens. TUNEL positive cells were observed in large amounts in cataract group, while rarely in normal lens group (Fig. 2A–D). Furthermore, the higher cell apoptosis rate was detected in cataract group than normal lens group (Fig. 2E).

3.3. Assessment of lens epithelial cell apoptosis induced by UV irradiation

In order to induce lens epithelial cell apoptosis, we used UVB irradiation and the SRA01/04 cell line. According to the preliminary experiment, the UV dosage totally acquired was 540 mJ/cm^2 with exposure duration of 25 min and intensity of 360 μW/cm^2. Cell apoptosis rate was increased by approximately 48.90% under this condition (Fig. 3A).

3.4. MiR-125b was downregulated during lens epithelial cell apoptosis and hindered apoptosis as induced by UV irradiation in vitro

The expression of miR-125b in lens epithelial cell apoptosis as induced by UV irradiation was detected using RT-qPCR. Lens epithelial cell apoptosis induced by UV irradiation showed significant downregulation of miR-125b compared with controls (SRA01/04 cell without UVB irradiation) (Fig. 3B).

Downregulated expression of miR-125b in both tissues and lens epithelial cell apoptosis induced by UV irradiation suggest that miR-125b

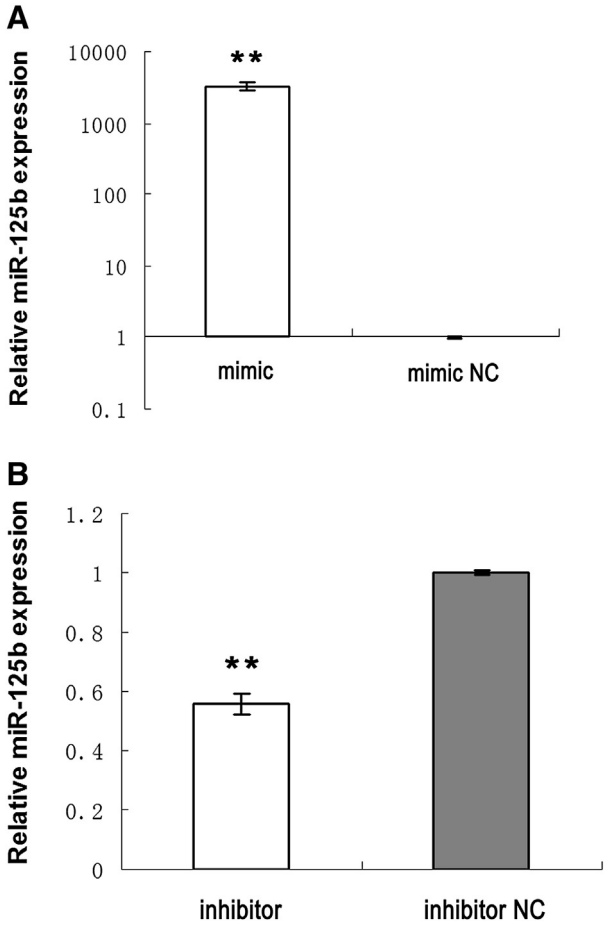

Fig. 4. Assessing transfection efficiency in lens epithelial cell apoptosis induced by UV irradiation. (A) The expression of miR-125b transfected with miR-125b mimic was increased. (B) The expression of miR-125b was significantly decreased when transfected with miR-125b inhibitor ($n = 3$, mean ± SD, $^{**}P < 0.001$).

is implicated in apoptosis. Thus, the next experiment was conducted to determine the potential effects of miR-125b on apoptosis in human lens epithelial cells. Before UV irradiation, the lens epithelial cell was transfected with miR-125b mimic, mimic control, inhibitor and inhibitor control. After 48 h, the expression of miR-125b was examined to identify transfection effects using RT-qPCR. The relative expression level of miR-125b in lens epithelial cell apoptosis induced by UV irradiation transfected with miR-125b mimic was increased (Fig. 4A), whereas such expression level was significantly decreased in cells transfected with miR-125b inhibitor (Fig. 4B).

As assayed by flow cytometry, the apoptosis rate of cells transfected with miR-125b mimic was reduced dramatically compared with controls (Fig. 5A). On the contrary, the apoptosis rate was increased markedly in the miR-125b inhibitor group (Fig. 5B).

3.5. MiR-125b targets p53

Three different target prediction software were used to predict potential target genes of miR-125b in SRA01/04 cells: TargetScan 5.2, Diana-microT and miRanda. Among the target gene list, we selected p53 which has three predicted binding sites with miR-125b for further analysis due to its important role in regulating cell proliferation [26] and apoptosis in response to DNA injury [27,28].

To determine whether miR-125b targets p53 in tissue specimens of anterior lens capsule of age-related cataract, 54 fresh anterior lens capsules of age-related cataract were selected with an equal number of normal anterior lens capsule specimens. The expression level of p53 mRNA and protein was determined using RT-qPCR and western blot. Both p53 mRNA and protein were remarkably higher in anterior lens capsules of age-related cataract (Fig. 6A and B), in contrast to a lower miR-125b level in the same tissue specimens (Fig. 1), demonstrating a reciprocal relationship between miR-125b and p53 expression.

To determine whether p53 is regulated by miR-125b, we transfected lens epithelial cell apoptosis *in vitro* with miR-125b mimic, mimic control, miR-125b inhibitor and inhibitor control before UV irradiation. At 48 h post transfection, RT-qPCR was performed to assess the mRNA expression level of p53. Overexpression of miR-125b led to a remarkable decrease in p53 mRNA compared with controls, while inhibition of miR-125b increased the level of p53 mRNA (Fig. 7A and B). As determined by western blot, high expression of miR-125b significantly decreased p53 protein compared with controls, whereas reduction of miR-125b resulted in an increase of p53 protein (Fig. 7C and D). These results suggested that the expression of p53 was regulated at both mRNA and protein levels by miR-125b *in vitro*.

To confirm if p53 is a direct target of miR-125b, luciferase activity assays were performed. The p53-3′-UTR was cloned into a luciferase reporter plasmid, and the luciferase reporter plasmid pmiR-RB-REPORT™-p53-3′-UTR or a mutant reporter plasmid carrying point mutations in the presumed miR-125b binding sites (pmiR-RB-REPORT™-mut-p53-3′-UTR) was co-transfected with miR-125b mimics. Then the luciferase activity at 48 h post transfection was examined. The luciferase activity was suppressed by approximately half in

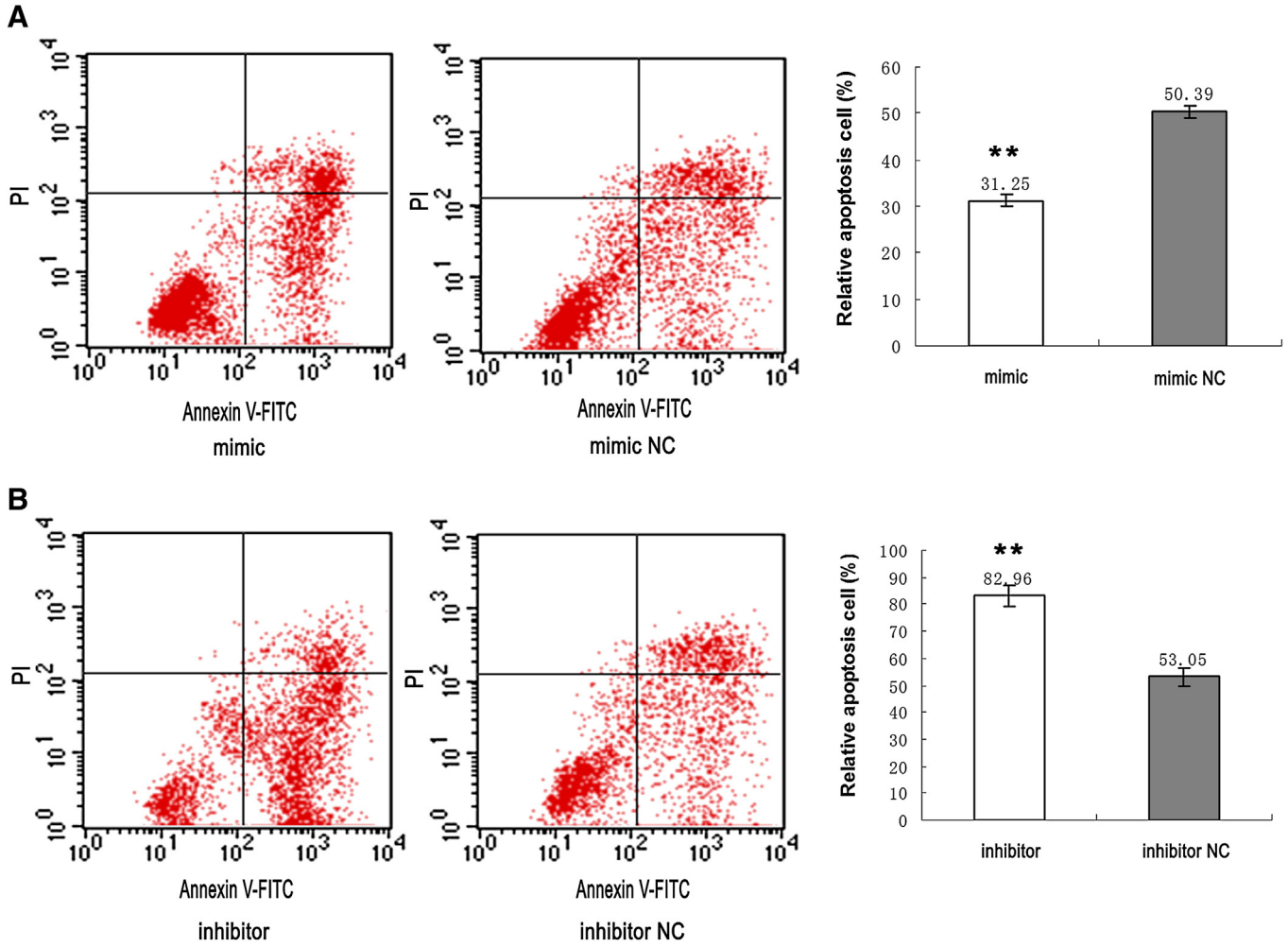

Fig. 5. The effect of miR-125b on apoptosis in lens epithelial cells. (A) Cell apoptosis rate as assayed by flow cytometry transfected with miR-125b mimic was reduced dramatically. (B) Cell apoptosis rate was increased markedly in the miR-125b inhibitor group ($n = 3$, mean ± SD, $^{**}P < 0.001$).

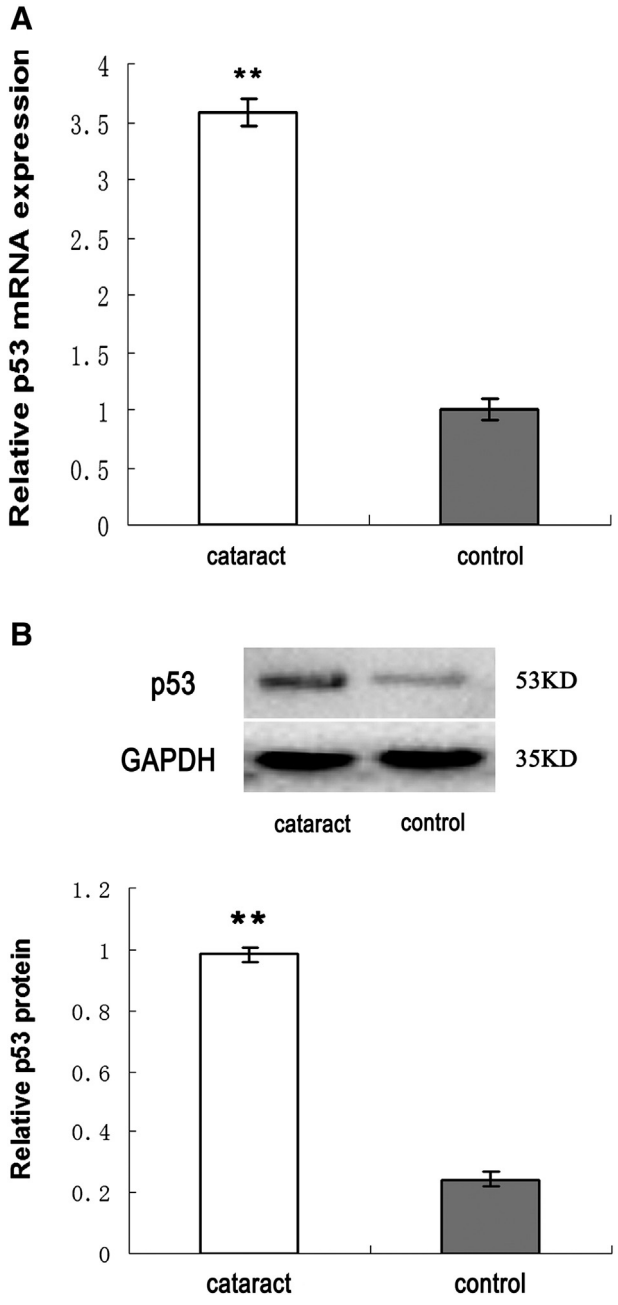

Fig. 6. P53 was upregulated in anterior lens capsule of age-related cataract. (A) Upregulated level of p53 mRNA was detected using RT-qPCR. (B) The expression of p53 protein detected by western blot was upregulated ($n = 3$, mean ± SD, $^{**}P < 0.001$).

cells transfected with the reporter plasmid carrying the wild type p53-3′-UTR compared with controls (Fig. 8). Nevertheless, no obvious suppression was observed when the reporter plasmid carried a mutant p53-3′-UTR (Fig. 8). These results indicated that miR-125b bound to p53 3′-UTR region directly and suppressed p53 expression.

To further confirm if miR-125b regulates cell apoptosis by targeting p53, the cell apoptosis rate was assessed when miR-125b and p53 were knocked down simultaneously using miR-125b inhibitor and p53 siRNA respectively. Before UV irradiation, the lens epithelial cell was co-transfected with the following combinations: (a) siRNA normal control (NC)/miRNA inhibitor NC; (b) miR-125b inhibitor/siRNA NC; (c) p53 siRNA/miR-125b inhibitor; and (d) p53 siRNA/miRNA inhibitor NC. Forty-eight hours after transfection, we measured p53 protein expression using western blot and cell apoptosis rate by flow cytometry. As shown in Fig. 9A, the expression of p53 protein increased in cells co-transfected with (b) miR-125b inhibitor/siRNA NC, and markedly decreased in cells co-transfected with (d) p53 siRNA/miRNA inhibitor NC. In cells co-transfected with (c) p53 siRNA/miR-125b inhibitor, the expression of p53 showed little change compared with cells co-transfected with (a) siRNA NC/miRNA inhibitor NC. Moreover, cell apoptosis rates showed good correlation with the level of p53 protein expression (Fig. 9B). Taken together, these results proved that co-transfection of miR-125b inhibitor and p53 siRNA inhibited the effect of miR-125b on cell apoptosis and miR-125b was vital in the regulation of cell apoptosis by directly targeting p53.

4. Discussion

MiRNAs are known to be key players in the regulation of transcripts involved in processes as diverse as embryonic development, differentiation, cellular proliferation, apoptosis, metabolism and adaptation to environmental stress [10]. Over one third of human genes appear to be conserved miRNA targets [29]. Most diseases were found to have changes in miRNA expression profile. Mutations of miRNA and its target genes have been reported to cause a variety of diseases. Given the involvement of miRNA regulation in multiple cellular processes, it is not surprising that this process plays a part in complex, multifactorial and environmentally-influenced cellular processes such as human disease and cellular and organismal ageing [30].

Research of miRNAs in Ophthalmology is just at its early stage. Ryan et al. [31] demonstrated that there were more than 30 miRNAs expressed in cornea and 17 in lens of adult mouse. MiR-184 had the highest hybridization signal in the corneal and lens arrays. Frederikse et al. [32] provided evidence that miRNAs and Dicer were expressed in mammalian lenses. Hughes et al. [15] found that mutation in the seed region of miR-184 caused anterior polar cataract. Peng et al. [16] demonstrated that let-7 expression was significantly related to the severity of lens opacity and age of age-related cataract. Xu et al. [33] found that at least 78 miRNAs were expressed in adult mouse retina, some of which were associated with retinal development, circadian rhythm (miR-183), retinal pigment degeneration (miR-1, miR-133, miR-183), and macular degeneration (miR-31, miR-150). Wu et al. [34] investigated miRNA expression profiles in human lens samples and found several differences between the expression profiles of transparent and cataractous lenses. The top eight differentially expressed miRNAs included miR-184, let-7b, miR-923, miR-1826, miR-125b, miR-1308, miR-26a, and miR-638 in transparent samples, and miR-184, miR-1826, let-7b/c, miR-24, miR-23b, miR-923, and miR-23a in cataractous lenses.

As a newly recognized mechanism of gene expression regulation, the contributions of miRNAs to disease pathogenesis and clinical therapy of lens disease have not been well studied. To date, research in this area was limited to the detection of miRNA expression. No investigation of miRNAs has been published regarding regulation.

The present study identified, for the first time, the impact of miR-125b on age-related cataract.

UVR was shown to induce the apoptosis of lens epithelial cells, which was considered to be a common cellular pathway for non-congenital cataract development [35]. P53 gene was closely associated with the lens epithelial cell apoptosis [36]. In the lens, the increased p53 expression could be responsible for apoptosis induced by UVR [37]. Recent studies confirmed that miRNAs and p53 can form a complicated multi-level regulatory network [38].

Li et al. [7] indicated that cataract patients may have deficient defense systems against factors such as oxidative stress and UV at the onset of the disease. Such stress could trigger lens epithelial cell apoptosis that then might initiate cataract development. In their study, all of the cataract patients examined had a substantial percentage of apoptotic lens epithelial cells while normal human lenses of comparable age had very few such cells. Their observations suggested that lens epithelial cell apoptosis may be an early and critical event during cataract development, and a common cellular basis for initiation of noncongenital

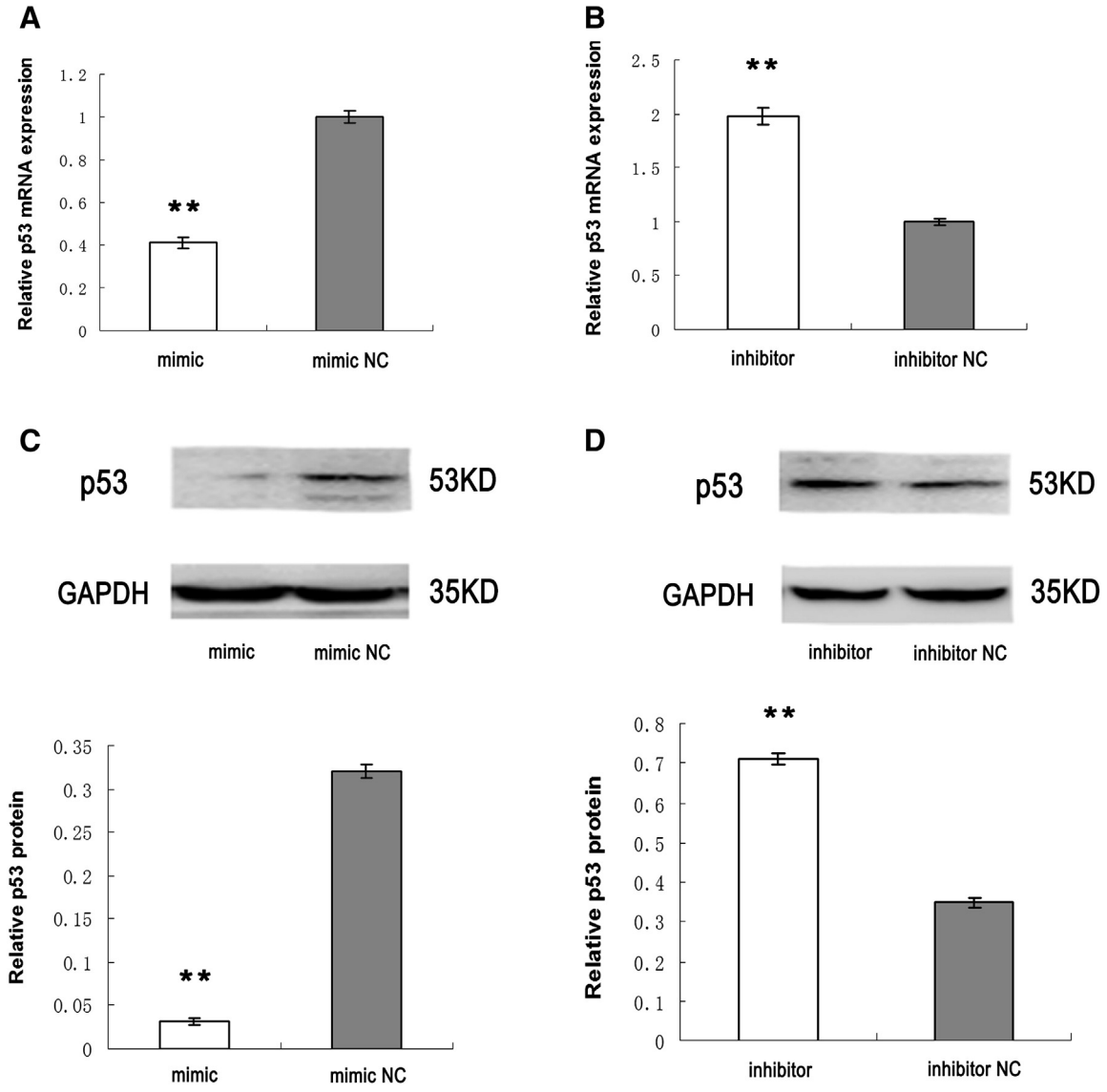

Fig. 7. The effect of miR-125b on p53 in lens epithelial cell apoptosis induced by UV irradiation. (A)&(B) Overexpression of miR-125b led to a marked decrease in p53 mRNA level, while inhibition of miR-125b increased the level of p53 mRNA. (C)&(D) High expression of miR-125b resulted in significant decrease of p53 protein, whereas reduction of miR-125b resulted in an increase of p53 protein ($n = 3$, mean ± SD, $^{**}P < 0.001$).

cataract formation. Andley *et al.* found that cells irradiated with monochromatic radiation at 297 nm was more than 6, 590, 1400 and 3000 times as effective in cell killing as 302, 325, 334 and 365 nm radiation, respectively [39]. UVB (280–320 nm) exposure is considered an important risk factor for cataract [40]. Consequently, we used UVB irradiation to induce SRA01/04 cell apoptosis.

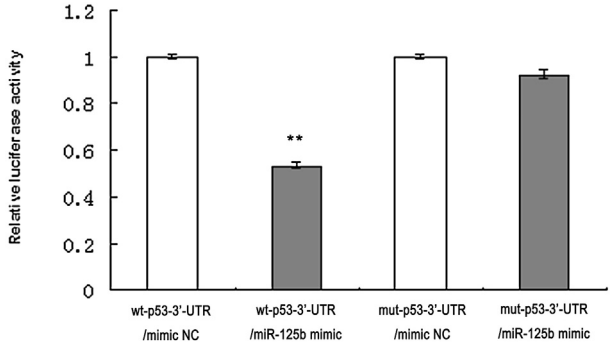

Fig. 8. P53 was a direct target of miR-125b in lens epithelial cell. MiR-125b binding sites in the 3′-UTR of p53 was assessed using luciferase activity assay. The luciferase activity was suppressed by approximately half in cells transfected with the reporter plasmid carrying the wild type p53-3′-UTR. No obvious suppression was observed when the reporter plasmid carried a mutant p53-3′-UTR ($n = 3$, mean ± SD, $^{**}P < 0.001$).

Currently, research on miR-125b is mostly concentrated in the field of cancer research. MiR-125b has high expression in certain tissues while low expression in others, indicating its relative tissue specificity. It is important to know that the same target may have different functions in different cellular processes, cellular context, and diseases. Therefore, targeting genes of specific cell-type may be useful as a more precise and specific therapy using miRNAs [20]. Zeng et al. [41] found that miR-125b modulated apoptosis in cancer cells by targeting p53. In our study, p53 was predicted as a potential target of miR-125b by three different target prediction software and subsequently confirmed by luciferase assay, for the first time, in SRA01/04 cells.

MiR-125b downregulation in age-related cataract lens tissues is also a novel finding. In addition, we demonstrated an inverse correlation between miR-125b and p53, consistent with the hypothesis that p53 is a target of miR-125b.

Using the human lens epithelial cell line (SRA01/04 cell) as an *in vitro* model, we found that miR-125b was downregulated during lens epithelial cell apoptosis. The involvement of miR-125b in apoptosis

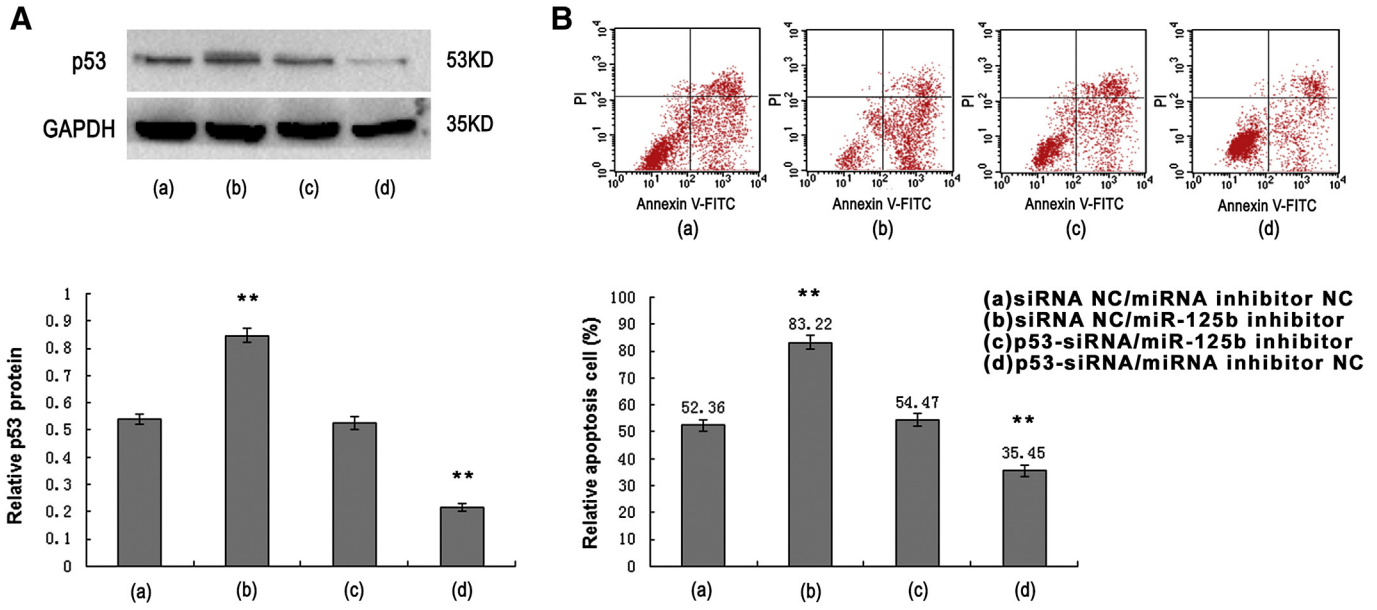

Fig. 9. MiR-125b regulated lens epithelial cell apoptosis by targeting p53. (A)&(B) The expression of p53 protein increased in cells co-transfected with (b) miR-125b inhibitor/siRNA NC, and markedly decreased in cells co-transfected with (d) p53 siRNA/miRNA inhibitor NC. In cells co-transfected with (c) p53 siRNA/miR-125b inhibitor, the expression of p53 showed little change compared with cells co-transfected with (a) siRNA NC/miRNA inhibitor NC. Cell apoptosis rates correlated well with the level of p53 protein expression ($n = 3$, mean ± SD, $^{**}P < 0.001$).

was confirmed by flow cytometry study, indicating that miR-125b is a suppressor of cell apoptosis. Moreover, we identified that p53 was a direct target, at least in part, of miR-125b in SRA01/04 cells by luciferase activity assay. All the data above suggested that miR-125b played a crucial role in cataract progression, and had the potential as a diagnostic and therapeutic tool of cataract.

Currently, a growing body of research elucidated that miRNA can be used as biomarkers for cancers and other diseases, depending on their abundance [20]. One milestone study [17] demonstrated the efficacy of a miR-26a-based therapy for liver cancer. Another important study [18] reported that therapeutic silencing of miR-122 played a crucial role in primates with chronic hepatitis C virus infection. Rayner et al. [19] showed that pharmacological inhibition of miR-33a and b was a promising therapeutic strategy to raise plasma HDL and lower VLDL triglycerides for the treatment of dyslipidemia. Intraocular injection of pre-miR-31, -150, or -184 significantly reduced ischemia-induced retinal neovascularization, and injection of pre-miR-31 or -150 also significantly reduced choroidal neovascularization, suggesting that alteration of miRNA levels contributes to two types of ocular neovascularization, and that injection or enhanced expression of miRNAs has the potential as a therapeutic strategy [42].

What is more, ongoing studies demonstrate the advantages using miR-125b as diagnostic and prognostic biomarkers. For example, miR-125b regulated Mcl-1 and IL6R, and was downregulated in hepatocellular carcinoma, confirming the tumor suppressive role of miR-125b in hepatocellular carcinoma and the potential usefulness of miR-125b in miRNA-based cancer therapy [43]. MiR-125b was significantly overexpressed in thyroid cancer and its level varies drastically between benign and malignant thyroid tissue specimens [44]. MiR-125b was directly involved in cancer progression and associated with poor prognosis in human colorectal cancer, suggesting that miR-125b could be an important prognostic indicator for colorectal cancer patients [45].

But unfortunately, little investigation of miRNA-based therapy for senescence disease has been reported at present.

In conclusion, miR-125b, a dramatically downregulated miRNA in age-related cataract lens tissue, plays a critical role in regulating human lens epithelial cell apoptosis at least in part by targeting p53. An upregulation of miR-125b may down regulate p53 and protect lens epithelial cells during cataract formation. Although further studies are required to elucidate the exact molecular mechanisms, it is certain that miR-125b is closely involved in the pathogenesis of cataract. The present study provides new insights into a potential diagnostic biomarker or even a therapeutic modality for cataract.

Acknowledgments

This project was supported by the National Natural Science Foundation of China (81270988, 81170836), the Natural Science Foundation of Liaoning Province, China (2013021095, 201202260), the Colleges and Universities Scientific Research Project of Liaoning Province, China (L2014305) and the Young Investigators Grant of the Fourth Affiliated Hospital of China Medical University (YB1217).

References

[1] D. Pascolini, S.P. Mariotti, G.P. Pokharel, R. Pararajasegaram, D. Etya'ale, A.D. Negrel, S. Resnikoff, Global update of available data on visual impairment: a compilation of population-based prevalence studies, Ophthalmic Epidemiol. 11 (2004) 67–115.
[2] N. Congdon, B. O'Colmain, C.C. Klaver, R. Klein, B. Munoz, D.S. Friedman, J. Kempen, H.R. Taylor, P. Mitchell, Causes and prevalence of visual impairment among adults in the United States, Arch Ophthalmol. 122 (2004) 477–485.
[3] S. West, Epidemiology of cataract: accomplishments over 25 years and future directions, Ophthalmic Epidemiol. 14 (2007) 173–178.
[4] S. Resnikoff, D. Pascolini, D. Etya'ale, I. Kocur, R. Pararajasegaram, G.P. Pokharel, S.P. Mariotti, Global data on visual impairment in the year 2002, Bull World Health Organ. 82 (2004) 844–851.
[5] F. Cruciani, F. Amore, G. Albanese, R. Anzidei, Investigation about causes of blindness and low vision among members of Blind and Visually Impaired Italian Union (UICI), Clin Ter. 162 (2011) e35–e42.
[6] WHO releases the new global estimates on visual impairmentAccessed on http://www.who.int/blindness/en/2011.
[7] W.C. Li, J.R. Kuszak, K. Dunn, R.R. Wang, W. Ma, G.M. Wang, A. Spector, M. Leib, A.M. Cotliar, M. Weiss, et al., Lens epithelial cell apoptosis appears to be a common cellular basis for non-congenital cataract development in humans and animals, J Cell Biol 130 (1995) 169–181.
[8] K. Chen, N. Rajewsky, The evolution of gene regulation by transcription factors and microRNAs, Nat Rev Genet. 8 (2007) 93–103.
[9] L. Peters, G. Meister, Argonaute proteins: mediators of RNA silencing, Mol Cell. 26 (2007) 611–623.
[10] D.P. Bartel, MicroRNAs: genomics, biogenesis, mechanism, and function, Cell. 116 (2004) 281–297.
[11] L. Maegdefessel, The emerging role of microRNAs in cardiovascular disease, J Intern Med (2014 Aug 27), http://dx.doi.org/10.1111/joim.12298 [Epub ahead of print].
[12] Y.H. Lee, S.Y. Kim, Y.S. Bae, Upregulation of miR-760 and miR-186 is associated with replicative senescence in human lung fibroblast cells, Mol Cell. 37 (2014) 620–627.

[13] S. Mimura, H. Iwama, K. Kato, K. Nomura, M. Kobayashi, H. Yoneyama, H. Miyoshi, J. Tani, A. Morishita, T. Himoto, A. Deguchi, T. Nomura, T. Sakamoto, K. Fujita, E. Maeda, K. Izuishi, K. Okano, Y. Suzuki, T. Masaki, Profile of microRNAs associated with aging in rat liver (Int J Mol Med), 34 (2014) 1065–1072.
[14] S. Gwee Sian Khee, Y.A. Mohd Yusof, S. Makpol, Expression of senescence-associated microRNAs and target genes in cellular aging and modulation by tocotrienol-rich fraction, Oxidative Med Cell Longev. (2014) 725929.
[15] A.E. Hughes, D.T. Bradley, M. Campbell, J. Lechner, D.P. Dash, D.A. Simpson, C.E. Willoughby, Mutation altering the miR-184 seed region causes familial keratoconus with cataract, Am J Hum Genet. 89 (2011) 628–633.
[16] C.H. Peng, J.H. Liu, L.C. Woung, T.J. Lin, S.H. Chiou, P.C. Tseng, W.Y. Du, C.K. Cheng, C.C. Hu, K.H. Chien, S.J. Chen, MicroRNAs and cataracts: correlation among let-7 expression, age and the severity of lens opacity, Br J Ophthalmol. 96 (2012) 747–751.
[17] J. Kota, R.R. Chivukula, K.A. O'Donnell, E.A. Wentzel, C.L. Montgomery, H.W. Hwang, T.C. Chang, P. Vivekanandan, M. Torbenson, K.R. Clark, J.R. Mendell, J.T. Mendell, Therapeutic microRNA delivery suppresses tumorigenesis in a murine liver cancer model, Cell. 137 (2009) 1005–1017.
[18] R.E. Lanford, E.S. Hildebrandt-Eriksen, A. Petri, R. Persson, M. Lindow, M.E. Munk, S. Kauppinen, H. Orum, Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection, Science. 327 (2010) 198–201.
[19] K.J. Rayner, C.C. Esau, F.N. Hussain, A.L. McDaniel, S.M. Marshall, J.M. van Gils, T.D. Ray, F.J. Sheedy, L. Goedeke, X. Liu, O.G. Khatsenko, V. Kaimal, C.J. Lees, C. Fernandez-Hernando, E.A. Fisher, R.E. Temel, K.J. Moore, Inhibition of miR-33a/b in non-human primates raises plasma HDL and lowers VLDL triglycerides, Nature. 478 (2011) 404–407.
[20] Y.M. Sun, K.Y. Lin, Y.Q. Chen, Diverse functions of miR-125 family in different cell contexts, J Hematol Oncol. 6 (2013) 6.
[21] M. Bousquet, D. Nguyen, C. Chen, L. Shields, H.F. Lodish, MicroRNA-125b transforms myeloid cell lines by repressing multiple mRNA, Haematologica. 97 (2012) 1713–1721.
[22] Q. Bi, S. Tang, L. Xia, R. Du, R. Fan, L. Gao, J. Jin, S. Liang, Z. Chen, G. Xu, Y. Nie, K. Wu, J. Liu, Y. Shi, J. Ding, D. Fan, Ectopic expression of MiR-125a inhibits the proliferation and metastasis of hepatocellular carcinoma by targeting MMP11 and VEGF, PLoS One. 7 (2012) e40169.
[23] N. Xu, L. Zhang, F. Meisgen, M. Harada, J. Heilborn, B. Homey, D. Grander, M. Stahle, E. Sonkoly, A. Pivarcsi, MicroRNA-125b down-regulates matrix metallopeptidase 13 and inhibits cutaneous squamous cell carcinoma cell proliferation, migration, and invasion, J Biol Chem. 287 (2012) 29899–29908.
[24] Y. Ge, Y. Sun, J. Chen, IGF-II is regulated by microRNA-125b in skeletal myogenesis, J Cell Biol. 192 (2011) 69–81.
[25] N. Ibaraki, S.C. Chen, L.R. Lin, H. Okamoto, J.M. Pipas, V.N. Reddy, Human lens epithelial cell line, Exp Eye Res. 67 (1998) 577–585.
[26] N.C. Reich, A.J. Levine, Growth regulation of a cellular tumour antigen, p53, in nontransformed cells, Nature. 308 (1984) 199–201.
[27] N. Kawagishi, Y. Hashimoto, H. Takahashi, A. Ishida-Yamamoto, H. Iizuka, Epidermal cell kinetics of pig skin in vivo following UVB irradiation: apoptosis induced by UVB is enhanced in hyperproliferative skin condition, J Dermatol Sci. 18 (1998) 43–53.
[28] Y. Li, C.L. Schlamp, G.L. Poulsen, M.W. Jackson, A.E. Griep, R.W. Nickells, p53 regulates apoptotic retinal ganglion cell death induced by N-methyl-D-aspartate, Mol Vis. 8 (2002) 341–350.
[29] B.P. Lewis, C.B. Burge, D.P. Bartel, Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets, Cell. 120 (2005) 15–20.
[30] L.W. Harries, MicroRNAs as mediators of the ageing process, Genes (Basel). 5 (2014) 656–670.
[31] D.G. Ryan, M. Oliveira-Fernandes, R.M. Lavker, MicroRNAs of the mammalian eye display distinct and overlapping tissue specificity, Mol Vis. 12 (2006) 1175–1184.
[32] P.H. Frederikse, R. Donnelly, L.M. Partyka, miRNA and Dicer in the mammalian lens: expression of brain-specific miRNAs in the lens, Histochem Cell Biol. 126 (2006) 1–8.
[33] S. Xu, P.D. Witmer, S. Lumayag, B. Kovacs, D. Valle, MicroRNA (miRNA) transcriptome of mouse retina and identification of a sensory organ-specific miRNA cluster, J Biol Chem. 282 (2007) 25053–25066.
[34] C. Wu, H. Lin, Q. Wang, W. Chen, H. Luo, H. Zhang, Discrepant expression of microRNAs in transparent and cataractous human lenses, Invest Ophthalmol Vis Sci. 53 (2012) 3906–3912.
[35] X. Sun, W. Zou, C. Zhao, Expression of P53 during lens epithelial cell apoptosis induced by ultraviolet, J Tongji Med Univ. 21 (2001) 263–264.
[36] J.C. Geatrell, P.M. Gan, F.C. Mansergh, L. Kisiswa, M. Jarrin, L.A. Williams, M.J. Evans, M.E. Boulton, M.A. Wride, Apoptosis gene profiling reveals spatio-temporal regulated expression of the p53/Mdm2 pathway during lens development, Exp Eye Res. 88 (2009) 1137–1151.
[37] M. Ayala, H. Strid, U. Jacobsson, P.G. Soderberg, p53 expression and apoptosis in the lens after ultraviolet radiation exposure, Invest Ophthalmol Vis Sci. 48 (2007) 4187–4191.
[38] Z. Feng, C. Zhang, R. Wu, W. Hu, Tumor suppressor p53 meets microRNAs, J Mol Cell Biol. 3 (2011) 44–50.
[39] U.P. Andley, J.G. Weber, Ultraviolet action spectra for photobiological effects in cultured human lens epithelial cells, Photochem Photobiol. 62 (1995) 840–846.
[40] T.W. Bochow, S.K. West, A. Azar, B. Munoz, A. Sommer, H.R. Taylor, Ultraviolet light exposure and risk of posterior subcapsular cataracts, Arch Ophthalmol. 107 (1989) 369–372.
[41] C.W. Zeng, X.J. Zhang, K.Y. Lin, H. Ye, S.Y. Feng, H. Zhang, Y.Q. Chen, Camptothecin induces apoptosis in cancer cells via microRNA-125b-mediated mitochondrial pathways, Mol Pharmacol. 81 (2012) 578–586.
[42] J. Shen, X. Yang, B. Xie, Y. Chen, M. Swaim, S.F. Hackett, P.A. Campochiaro, MicroRNAs regulate ocular neovascularization, MolTher. 16 (2008) 1208–1216.
[43] H.Y. Jia, Y.X. Wang, W.T. Yan, H.Y. Li, Y.Z. Tian, S.M. Wang, H.L. Zhao, MicroRNA-125b functions as a tumor suppressor in hepatocellular carcinoma cells, Int J Mol Sci. 13 (2012) 8762–8774.
[44] M.R. Vriens, J. Weng, I. Suh, N. Huynh, M.A. Guerrero, W.T. Shen, Q.Y. Duh, O.H. Clark, E. Kebebew, MicroRNA expression profiling is a potential diagnostic tool for thyroid cancer, Cancer. 118 (2012) 3426–3432.
[45] N. Nishida, T. Yokobori, K. Mimori, T. Sudo, F. Tanaka, K. Shibata, H. Ishii, Y. Doki, H. Kuwano, M. Mori, MicroRNA miR-125b is a prognostic marker in human colorectal cancer, Int J Oncol. 38 (2011) 1437–1443.