Clinical Surgery Research Communications

Clin Surg Res Commun 2019; 3(2): **16-24**

DOI: 10.31491/CSRC.2019.06.032

Research article

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CASC15 Promotes Lens Epithelial Cell Apoptosis in Age-Related Cataracts by Regulating MiR-139-5p/caspase-3

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Abstract

Objective: The aim of this study was to reveal the role of CASC15 and miR-139-5p function in regulating age-related cataracts (ARC).

Methods: The expression of CASC15, miR-139-5p and caspase-3 were detected by qRT-PCR. Western blot was used to analyze the level of apoptosis-related proteins. Flow cytometry was used to measure the apoptosis of SAR01/04l cells treated with UV irradiation.

Results: The expression of CASC15 and caspase-3 in the lens of ARC was higher than that in normal lens, but the expression of miR-139-5p had opposite trend. The level of caspase-3 and Bax were increased and Bcl-2 was decreased in ARC group. In addition, CASC15 negatively regulated miR-139-5p expression and promoted SAR01/04l cell apoptosis. CASC15 could combine with miR-139-5p, and miR-139-5p could combine with caspase-3 3'-UTR. Furthermore, pcDNA-caspase-3 could reverse the inhibitory effect of si-CASC15 and miR-139-5p mimic on SRA01/04 cell apoptosis.

Conclusion: CASC15 promoted lens epithelial cell apoptosis by regulating miR-139-5p/caspase-3.

Keywords: ARC; CASC15; LECs apoptosis; miR-139-5p

INTRODUCTION

Cataract is a common eye disease, which is the leading cause of visual impairment and blindness [1,2]. Age-related cataract (ARC) is the most common types of cataracts, which often occurs in people aged 50 years and over, and is characterized by loss of lens transparency [3]. Recently, with the aging of the population intensified, the incidence of ARC has increased, and most of the new cases come from low-income countries [4]. ARC is a complex multifactorial disease caused by the interaction of genes and environmental factors [5]. Several studies found that risk factors such as age, sex, smoking, diabetes, sunlight or ultraviolet B radiation, vitamins and body mass index (BMI) were related to the occurrence and development of ARC [6]. In addition, genetic background also plays an important role in the development of ARC [7]. At present, the surgical treatment of ARC has achieved good results worldwide, but the etiology of ARC is not clearly understood. A large number of studies have shown that the apoptosis of lens epithelial cells (LECs) is the common cytological basis for the formation of cataracts except congenital cataract [8]. Therefore, to investigate the mechanism of LECs apoptosis is important for the prevention and treatment of ARC.

Long non-coding RNA (lncRNA) is a non-coding RNA with a length greater than 200 nucleotides [9]. Studies have shown that lncRNAs play an important role in Dosage compensation effect, epigenetic regulation, cell cycle regulation and cell differentiation regulation [10]. Moreover, lncRNAs are closely related to the development of tumor [11]. Cancer susceptibility candidate 15 (CASC15) is a lncRNA and expresses in brain [12]. Recently, studies have suggested that CASC15 played an important role in neuroblastoma [12] and melanoma [13], etc, but the role of CASC15 in LECs apoptosis has not been reported.

MicroRNAs (miRNAs) are endogenously expressed non-coding RNAs, which are widely involved in cell proliferation, apoptosis and autophagy [14]. Recent studies have shown that miRNAs play an important role in the regulation of LECs apoptosis [15]. Chen et al. [16] found that miR-34a could promote LECs apoptosis through E2F3 pathway. Zhang et al. [17] suggested that downregulation of miR-133b could inhibit LECs apoptosis. MiR-139-5p is a relatively widespread miRNA in human cells. It has been found that miR-139-5p can be used as a biomarker for cancer [18]. Chen et al. [19] found that miR-139-5p inhibited cell proliferation of uterine fibroids. Song et al.

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Received: 25 March 2019 Accepted: 28 May 2019

[20] reported that miR-139-5p inhibited the metastasis of colorectal cancer. However, the effects of miR-139-5p on the apoptosis of LECs and its regulation mechanism have not been reported. LncBase Predicted v.2 software predicted that CASC15 combined with miR-139-5p, and TargetScan software predicted that miR-139-5p could combine with 3'-UTR of caspase-3. Caspase-3 is an important factor for cell apoptosis. Previous studies have demonstrated that caspase-3 plays a key role in LECs apoptosis [21,22]. Thus, we speculated that the role of CASC15 and miR-139-5p on LECs apoptosis might be related with caspase-3.

In this study, we first observed the expression of CASC15, miR-139-5p and apoptosis-related proteins in the lens tissues of patients with ARC and SRA01/04 cells, and then investigated the regulation mechanism of CASC15/miR-139-5p/caspase-3 on LECs apoptosis. The aim of this study was to reveal the relationship between CASC15 and ARC, and to provide a new target for the prevention and treatment of blinding eye disease.

MATERIALS AND METHODS

Samples

In this study, we selected 25 ARC patients who had been excluded other eye diseases. It included 15 males and 10 females, and with the average age of 60.7±5.3 years (aged from 50 to 70). In all cases, continuous curvilinear capsulorhexis was used to obtain the anterior capsule of the lens in cataract surgery. Then, eye anterior lens capsules transparent specimens of 25 adults without cataract were collected as the control group. It included 14 males and 11 females, and with the average age of 59.2±5.4 years (aged from 50 to 70). These lenses from postmortem eyes were obtained with 8 to 24 hours after death. These specimens were provided by the Miyun Teaching Hospital Affiliated to Capital Medical University. The study protocol was approved by the Ethics Committee of the Miyun Teaching Hospital Affiliated to Cap-

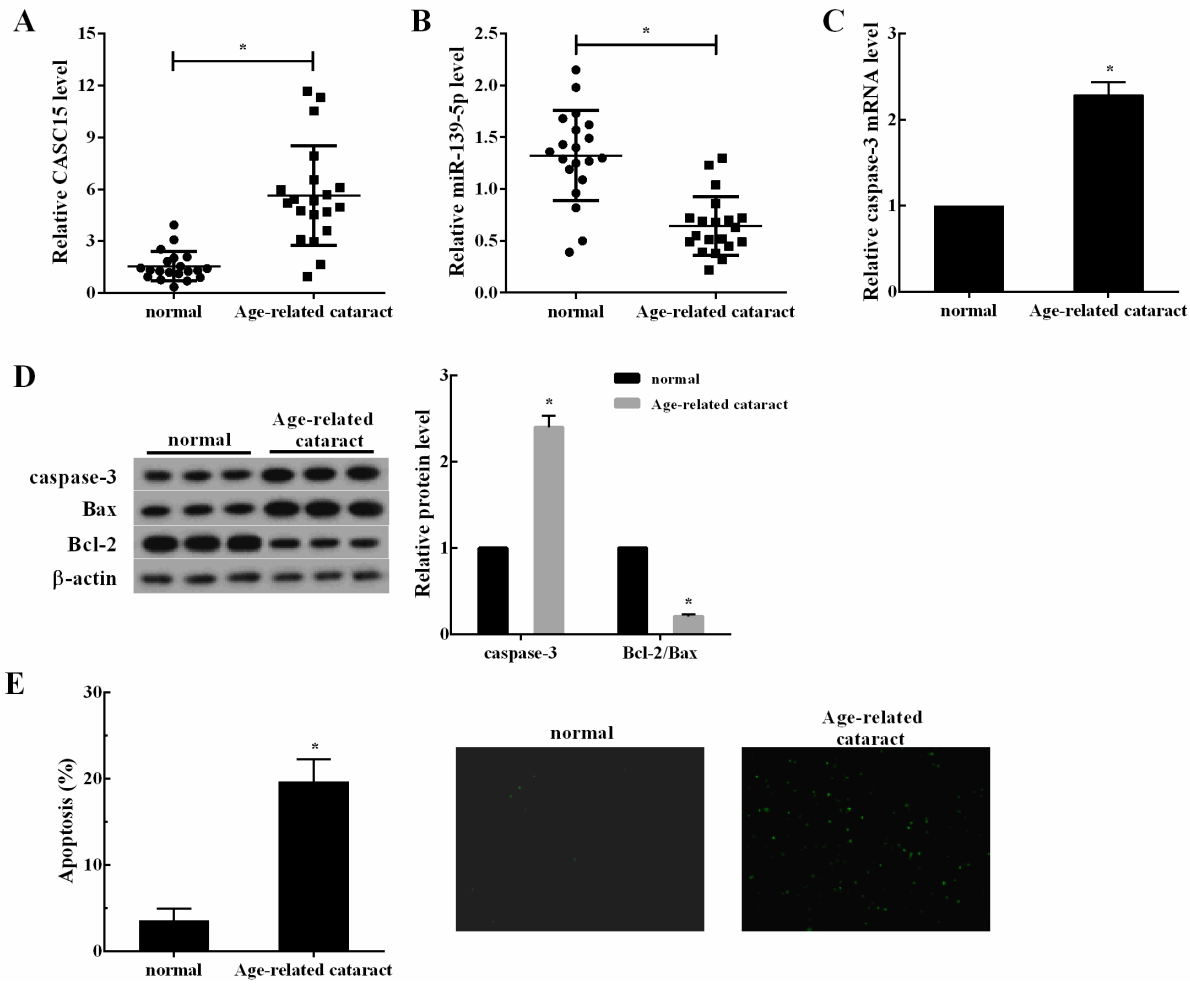

Figure 1. The expression of CASC15, miR-139-5p and apoptosis-related protein in the crystalline lens of ARC. **(A)** CASC15 expression. **(B)** miR-139-5p expression. **(C)** The mRNA expression of caspase-3. **(D)** The protein expression of caspase-3, Bax and Bcl-2. **(E)** Cell apoptosis. *P<0.05, vs control group.

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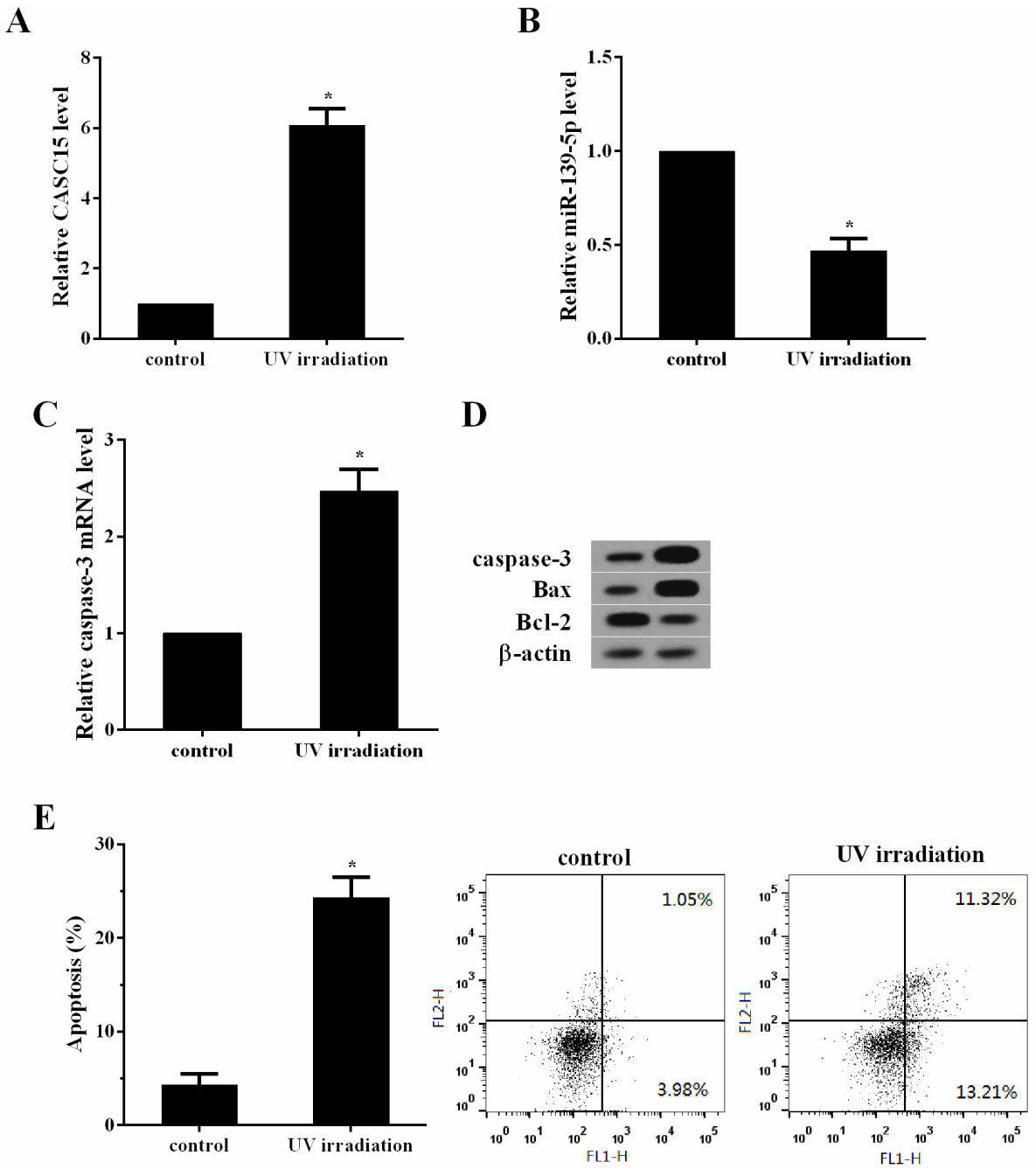

Figure 2. The expression of CASC15, miR-139-5p and apoptosis-related protein in SRA01/04 cell model. **(A)** CASC15 expression. **(B)** miR-139-5p expression. **(C)** The mRNA expression of caspase-3. **(D)** The protein expression of caspase-3, Bax and Bcl-2. **(E)** Cell apoptosis. *P<0.05, vs control group.

ital Medical University, and all patients were informed consent.

Cell culture

Human LECs line - SRA01/04 cells (purchased from the cell bank of Chinese Academy of Medical Sciences) were cultured in DMEM medium (Gibco, USA) supplemented with 10% FBS (Gibco, USA), 100 U/ml of penicillin and 100 μg/ml of streptomycin, and incubated at 37°C in an incubator with 5% CO_2.

Ultraviolet (UV) irradiation

Apoptosis model of HLEC was established by ultraviolet irradiation. The cells were irradiated with an ultraviolet (UV) lamp (XX-15B, Spectroline, Westbury, USA) [23]. The irradiation intensity was 360 μW/cm^2, and the irradiation time was 30min.

Cell transfection

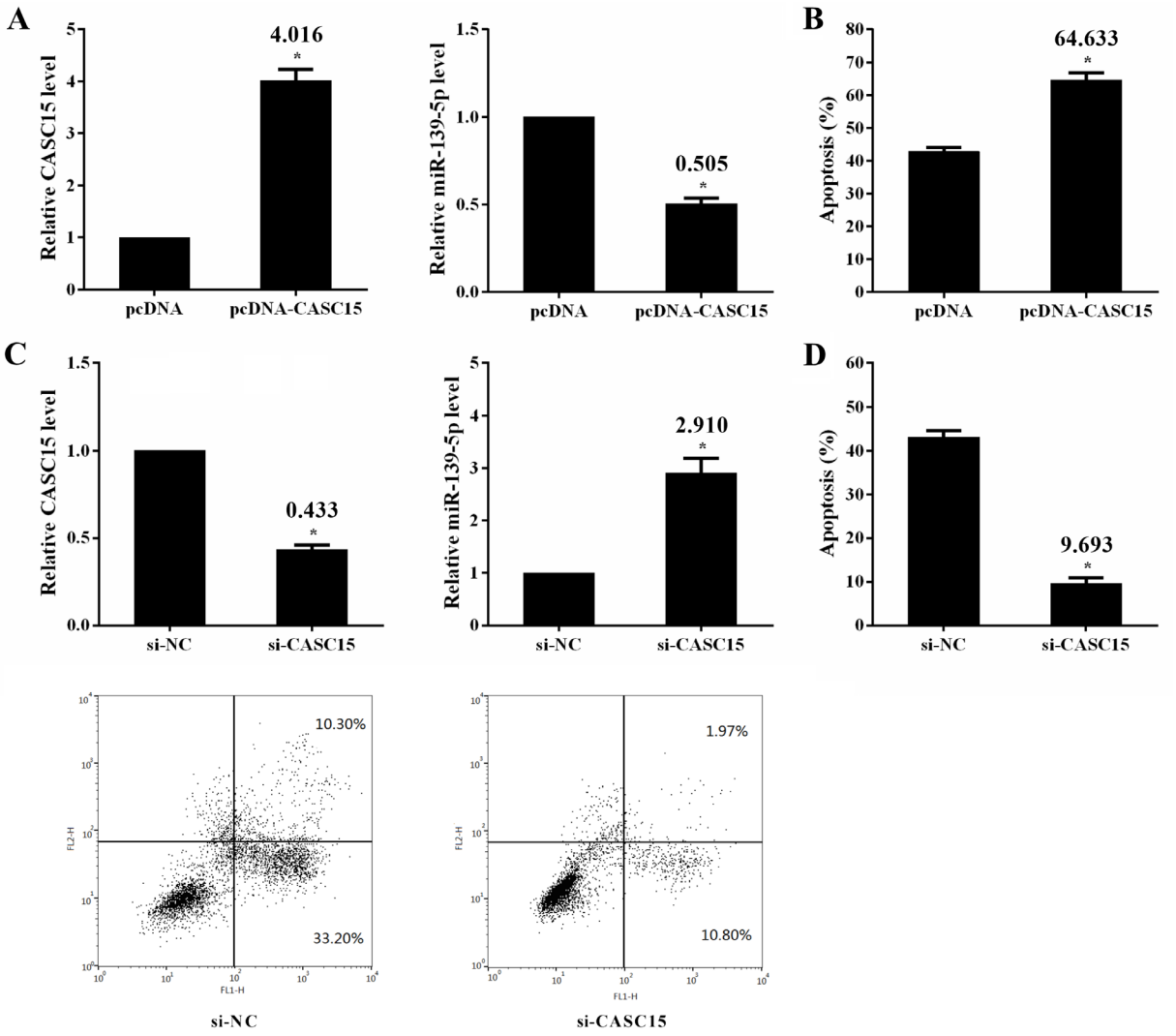

Figure 3. The effect of CASC15 on SRA01/04 cell apoptosis. The SRA01/04 cells were transfected with pcDNA, pcDNA-CASC15, si-NC and si-CASC15. **(A)** The expression of CASC15 and miR-139-5p in SRA01/04 cells transfected with pcDNA and pcDNA-CASC15. **(B)** Cell apoptosis. **(C)** The expression of CASC15 and miR-139-5p in SRA01/04 cells transfected with si-NC and si-CASC15. **(D)** Cell apoptosis. *P<0.05, vs pcDNA or si-NC.

Si-CASC15, si-caspase-3, si-NC, pcDNA-CASC15, pcDNA, miR-139-5p inhibitor and inhibitor-NC were respectively transfected into SRA01/04 cells using Lipofectamine 2000 (Invitrogen, USA) following the manufacturer's instructions.

Quantitative real-time PCR (qRT-PCR)

According to the manufacturer's instructions, the total RNA was extracted with Trizol Kit (Invitrogen, USA), and then reverse transcribed into cDNA for qRT-PCR. The expression of CASC15, miR-139-5p and caspase-3 were measured using the $2^{-\Delta\Delta Ct}$ method.

The primer sequences:

MiR-139-5p F: 5'-TCTACAGTGCACGTGTCTCCAGT-3'
MiR-139-5p R: 5'-TGGAGACACGTGCACTGTAGATT-3'
Caspase-3 F: 5'-TAC CTC AAC TTC GAC ATC AGG-3'
Caspase-3 R: 5'-CAG TGG AAA AGC TGT ACT GC-3'
CASC15 F: 5'-CGCCGGGGTATCTCCCTCTCG-3'
CASC15 R: 5'-CATTTCCCCCGCTGCAGTCCA-3'.

Western blot

Total protein was extracted using the protein lysis buffer (Solarbio, Beijing). Then, the protein samples were resolved by 10% SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, USA). The membranes were incubated with the primary antibodies against caspase-3 (1: 500, Abcam, Chicago, IL, USA), Bax (1: 1000, Abcam), Bcl-2 (1: 1000, Abcam) and β-actin (1:1000, Abcam) overnight at 4°C, and then incubated with secondary antibody peroxidase conjugated goat rabbit anti-IgG (Solarbio, Beijing). Quantity one software was used to quantify the band

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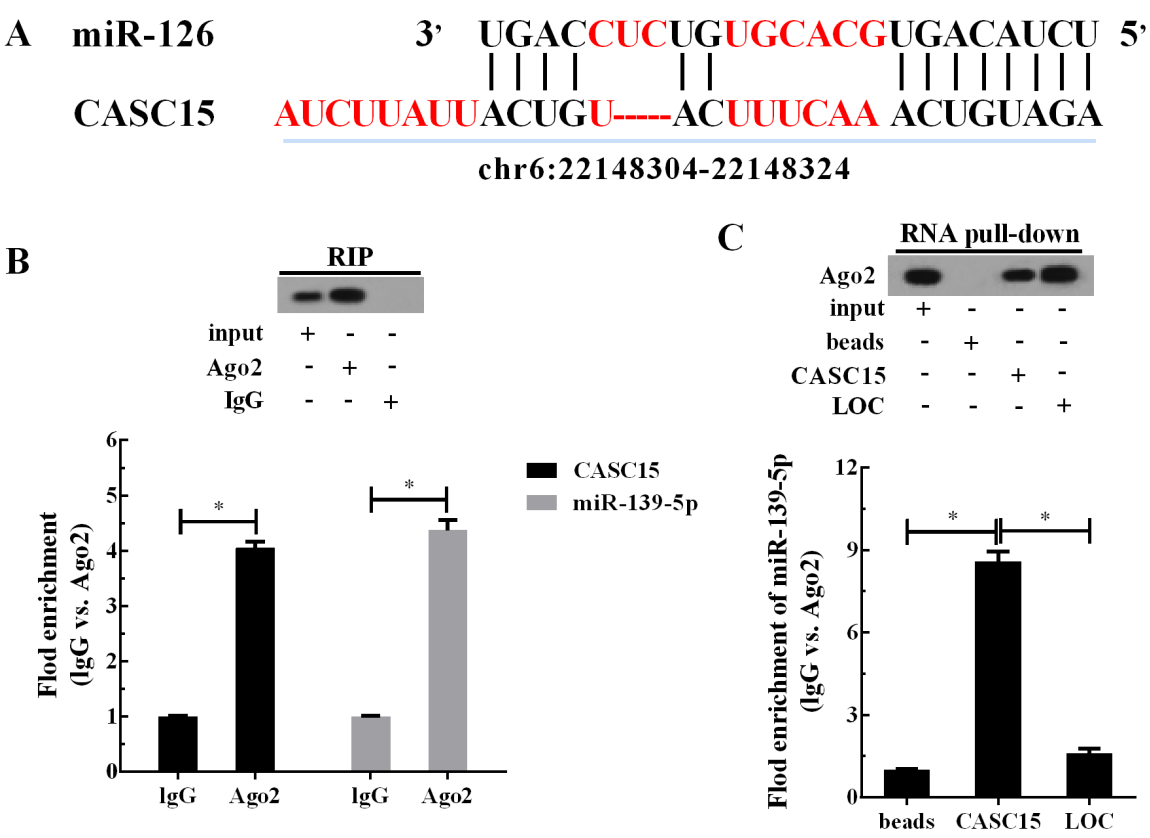

Figure 4. CASC15 combined with miR-139-5p. **(A)** The software (LncBase Predicted v.2) predicted binding sites between CASC15 and miR-139-5p. **(B)** The accumulation of CASC15 and miR-139-5p in Ago2. Ago2 was detected using IP-western. The expression of CASC15 and miR-139-5p was analyzed by qRT-PCR. **(C)** Pull-down of Ago2 by CASC15 or Loc was measured by western blotting, and miR-139-5p in the RNA-precipitated samples was measured by qRT-PCR. *P<0.05, vs IgG or beads.

intensity.

TUNEL assay

Terminal dexynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was used to measure cell apoptosis in tissue samples [24]. Paraffin-embedded eye anterior lens capsules transparent specimens were cut into tissue sections of 5 μm, and fixed in 4% (w/v) paraformaldehyde. Before the TUNEL assay, the samples were permeated with 0.1% (v/v) TritonX-100 (Beyotime, Shanghai, China), and blocked with 3% (v/v) H_2O_2. The TUNEL reaction mixture was then dropped onto the surface of the samples. After incubating at 37°C in a dark and humid environment for one hour, converter-POD was dropped onto the surface of the samples, and incubated for 30 min at 37°C followed by diaminobenzidine substrate. The samples were counterstained with hematoxylin, analyzed under a fluorescence microscope. The percentage of TUNEL-positive cells was used to calculate the apoptosis rate.

Flow cytometry analysis

Cell apoptosis was detected using flow cytometry analysis. According to the manufacturer's instructions, Annexin V-EGFP/PI (apoptosis detection kit, Research Science, Shanghai) was used to measure cells apoptosis. The samples were analyzed by flow cytometry (Becton Dickinson, CA). Then, the data were analyzed using Cell Quest software.

Luciferase reporter assay

The miR-139-5p binding site of caspase-3 was amplified by PCR. Then, caspase-3 3'UTR fragment or mutant caspase-3 3'UTR fragment were cloned into vector pcDNA with a firefly luciferase reporter gene (Beyotime, China). The miR-139-5p mimic or inhibitor or negative controls (NC) were respectively transfected into SRA01/04 cells. After 48h transfection, the cells were collected for measurement of luciferase activity.

RNA-binding protein immunoprecipitation (RIP) assay

According to the manufacturer's instructions, RIP assay was performed using RIP Kit (Sigma-Aldrich, Shanghai). The cells were lysed in RIP buffer and incubated with anti-Ago2 antibody (Sigma-Aldrich, Shanghai). IgG was used as a negative control. Trizol reagent (Invitrogen,

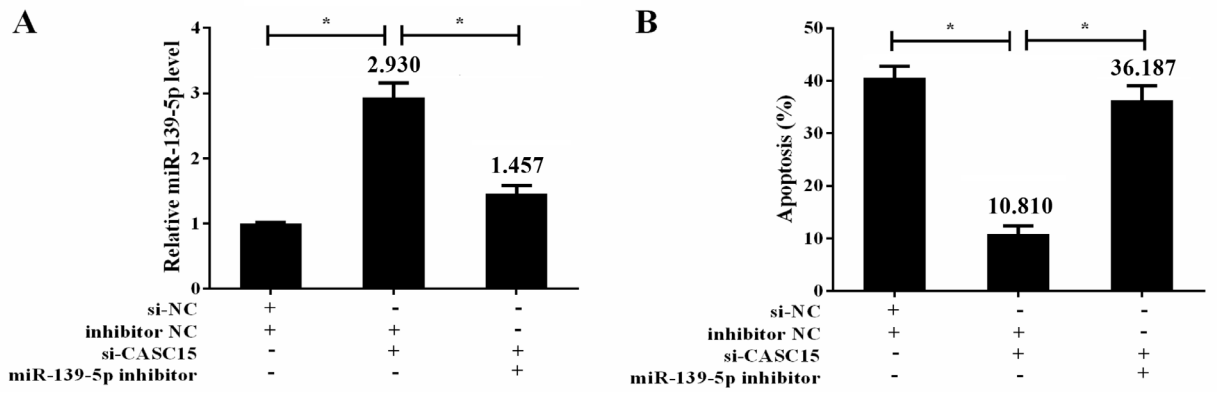

Figure 5. The effect of CASC15 on the expression of miR-139-5p in SRA01/04 cells. The SRA01/04 cells were divided into three groups, and co-transfected with si-NC and inhibitor-NC, si-CASC15 and inhibitor-NC, si-CASC15 and miR-139-5p inhibitor, respectively. **(A)** miR-139-5p expression. **(B)** Cell apoptosis. *P<0.05, vs si-NC+inhibitor-NC or si-CASC15+inhibitor-NC.

USA) was used to extract precipitated RNA. The level of CASC15 and miR-139-5p was analyzed by qRT-PCR.

RNA pull-down assay

RNA pull-down assay was performed by Magnetic RNA-Protein Pull-Down Kit (Thermo Fisher Scientific, USA) following the manufacturer's instructions. Western blot was used to detect the level of Ago2, and qRT-PCR was used to identify the level of miR-139-5p.

Statistical analysis

The experiments were repeated at least three times and analyzed by using SPSS 19.0 software. The data were presented as mean ± standard deviation (SD). P<0.05 was considered as statistical significance.

RESULTS

The expression of CASC15, miR-139-5p and apoptosis-related protein in the crystalline lens of ARC

qRT-PCR results showed that the CASC15 expression in the crystalline lens of ARC was markedly higher than that in the normal crystalline lens (Figure 1A), but the expression of miR-139-5p had an opposite trend (Figure 1B). Moreover, the mRNA expression of caspase-3 was increased significantly in the crystalline lens of the ARC compared with the normal crystalline lens (Figure 1C). The protein expression of caspase-3 and Bax were increased and Bcl-2 expression was decreased markedly in the ARC group compared with the normal group (Figure 1D). In addition, the cell apoptotic rate in ARC group was higher than that in normal group (Figure 1E).

The expression of CASC15, miR-139-5p and apoptosis-related protein in SRA01/04 cell model

As shown in Figure 2A&B, compared with the control cells, the expression of CASC15 in SRA01/04 cells treated with UV irradiation was markedly increased and the miR-139-5p expression was significantly decreased. Moreover, the mRNA expression of caspase-3 and the protein expression of caspase-3 and Bax were significantly increased in UV group, and Bcl-2 expression was significantly decreased compared with control group (Figure 2C&D). In addition, as shown in Figure 2E, UV irradiation markedly increased SRA01/04 cell apoptotic rate.

CASC15 promoted SRA01/04 cell apoptosis

The SRA01/04 cells were transfected with pcDNA, pcDNA-CASC15, si-NC and si-CASC15 respectively, and were treated with UV irradiation for 30mins. The results showed that pcDNA-CASC15 could promote the expression of CASC15 and inhibit the expression of miR-139-5p (Figure 3A). On the other hand, overexpression of CASC15 significantly promoted the apoptosis of SRA01/04 cells (Figure 3A&B). However, si-CASC15 reversed the trend. si-CASC15 could decrease CASC15 expression and increase miR-139-5p expression, and inhibit cell apoptosis (Figure 3C&D).

CASC15 combined with miR-139-5p

The software (LncBase Predicted v.2) predicted that CASC15 combined with miR-139-5p (Figure 4A). As shown in Figure 4B, Ago2 antibody precipitated the Ago2 protein from the cell lysates, and compared with IgG control, higher CASC15 and miR-139-5p in the Ago2 pellet was detected by qRT-PCR. Moreover, RNA pull-down assay showed that CASC15 interacted with Ago2 directly (Figure 4C). Compared with control, the content of miR-139-5p in CASC15 pulled down pellet was significantly increased, and the content of miR-139-5p in Loc (the positive control of CASC15) pulled down pellet was only slightly increased (Figure 4C).

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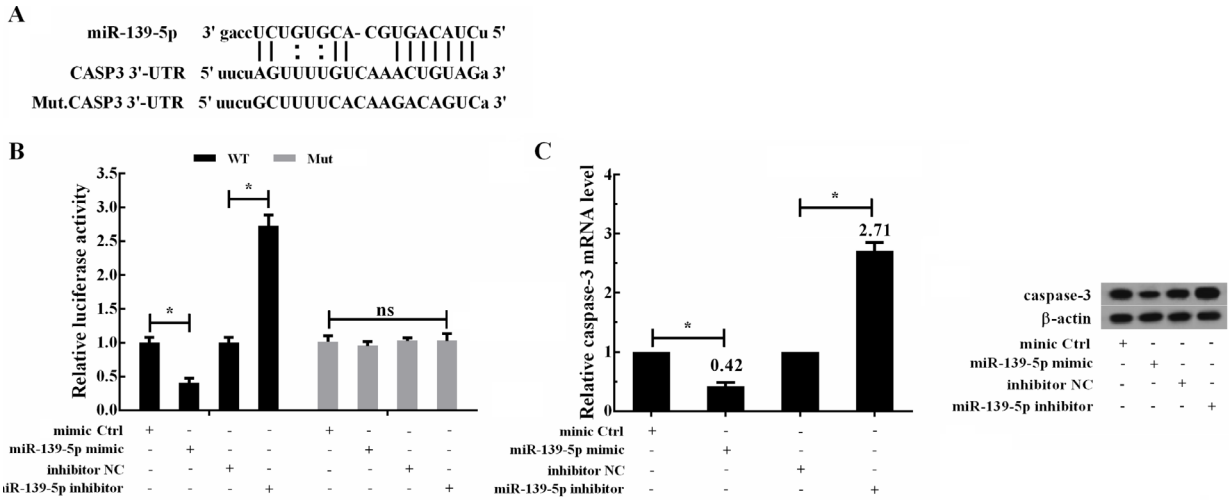

Figure 6. The effect of miR-139-5p on the expression of caspase-3 in SRA01/04 cells. **(A)** The software (TargetScan) predicted that miR-139-5p could combine with 3'-UTR of caspase-3. **(B)** The luciferase activity of 3'-UTR of caspase-3. **(C)** Caspase-3 expression. *P<0.05, vs mimic control or inhibitor NC.

CASC15 negatively regulated miR-139-5p expression in SRA01/04 cells

The SRA01/04 cells were divided into three groups, and co-transfected with si-NC and inhibitor-NC, si-CASC15 and inhibitor-NC, si-CASC15 and miR-139-5p inhibitor, respectively. The results showed that si-CASC15 up-regulated miR-miR-139-5p expression (Figure 5A) and inhibited SRA01/04 cell apoptosis (Figure 5B). However, miR-139-5p inhibitor could reverse the trend.

The effect of miR-139-5p on the expression of caspase-3

The software (TargetScan) predicted that miR-139-5p could combine with 3'-UTR of caspase-3 (Figure 6A). As shown in Figure 6B, in caspase-3'URT-WT group, miR-139-5p mimic could reduce the luciferase activity compared with mimic control, and miR-139-5p inhibitor could markedly enhance the luciferase activity compared with inhibitor control. However, the luciferase activity had no significant change in caspase-3'URT-MUT group. In addition, as shown in Figure 6C, SRA01/04 cells were divided into four groups: miR-139-5p mimic group, mimic control group, miR-139-5p inhibitor group, and inhibitor control group. The results showed that the mRNA and protein expression of caspase-3 in the miR-139-5p mimic group was decreased compared with the mimic control group, while the caspase-3 expression in the miR-139-5p inhibitor group was increased compared with the inhibitor control group.

CASC15 promoted apoptosis of SRA01/04 cells by regulating miR-139-5p/caspase-3

As shown Figure 7A, SRA01/04 cells were transfected with si-caspase-3, pcDNA-CASC15 and their controls. The results showed that the SRA01/04 cell apoptotic rate was increased in the pcDNA-CASC15 group compared with the pcDNA group, and decreased in the pcDNA-CASC15+si-caspase-3 group compared with the pcDNA-CASC15+si-NC group. As shown Figure 7B, SRA01/04 cells were transfected with pcDNA-caspase-3, miR-139-5p mimic, si-CASC15 and their controls. The results showed that the SRA01/04 cell apoptotic rate was increased in the pcDNA-caspase-3+si-CASC15 group compared with the pcDNA+si-CASC15 group, and the cell apoptotic rate was also increased in the pcDNA-caspase-3+miR-139-5p mimic group compared with the pcDNA+miR-139-5p mimic group. Moreover, compared with pcDNA+si-CASC15 and pcDNA+miR-139-5p mimic, the expression of caspase-3 and Bax in SRA01/04 cells transfected with pcDNA-caspase-3 was increased, but the Bcl-2 expression was decreased.

DISCUSSION

ARC, known as senile cataract, is the most common reason of blindness in elder people. Although the surgery is still the most effective treatment for cataract [25], the huge medical expenditure of surgical treatment also brings heavy burden to health resources and social economy of our country. Thus, it is imperative to explore new approaches to non-surgical treatment of cataract.

In this study, we showed that CASC15 and caspase-3 were up-regulated in lens tissues of ARC and UV irradiation-induced SAR01/04 cells, but miR-139-5p was down-regulated. Moreover, we found that overexpression CASC15 could decrease miR-139-5p expression and promote apoptosis of SAR01/04l cells. These results indicated that CASC15, miR-139-5p and caspase-3 might

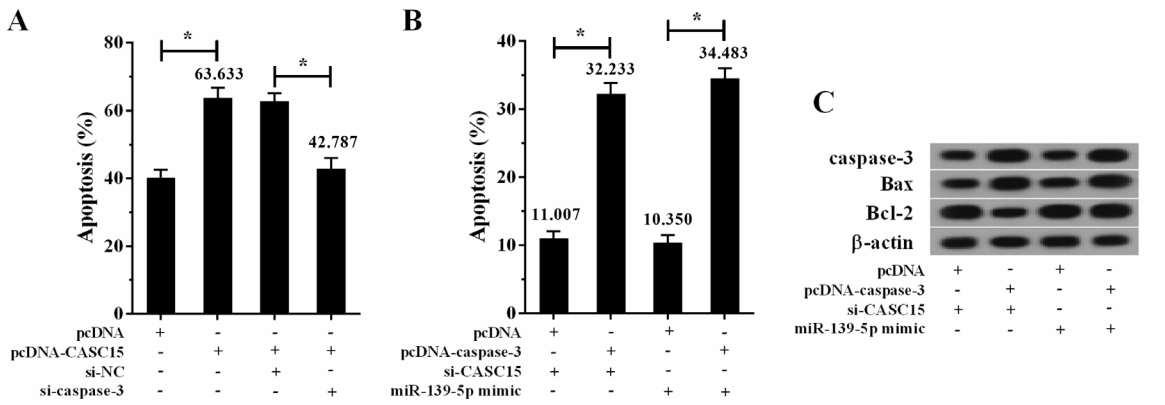

Figure 7. CASC15 affects apoptosis of SRA01/04 cells by regulating miR-139-5p/caspase-3. The SRA01/04 cells were transfected with pcDNA-CASC15, si-CASC15, si-caspase-3, pcDNA-caspase-3, miR-139-5p mimic and control vector, respectively. **(A&B)** Cell apoptosis. **(C)** The expression of caspase-3, Bax and Bcl-2. *P<0.05, vs pcDNA or pcDNA-CASC15+si-NC or pcDNA+si-CASC15 or pcDNA+miR-139-5p mimic.

be associated with LECs apoptosis in ARC. Therefore, we further studied the mechanism of CASC15, miR-139-5p and caspase-3 in ARC.

LncRNAs are associated with various eye diseases. Xu et al. [26] found that Vax2os1 and Vax2os2 were specifically expressed in intra-ocular retinal neovascularization. Yan et al. [27] reported that down-regulation of lncRNA-MIAT could reduce the formation of retinal neovascularization induced by diabetes. Young et al. [28] suggested that knockdown of TUG1 could inhibit the migration of developing rod cells in the outer nuclear layer. The present study found that CASC15 played an important role in ARC. Moreover, CASC15 could be combined miR-139-5p, and knockdown of CASC15 could increase miR-139-5p expression and inhibit SAR01/04l cell apoptosis. These results suggested CASC15 played a role in LECs apoptosis by regulating miR-139-5p expression.

In recent years, a lot of data reported that miRNAs played an important role in the development and progression of cataract. Hughes et al. [29] found that the mutation of miR-184 seed region could lead to anterior polar cataract. Qin et al. [30] reported that miR-125b inhibited LECs apoptosis in patients with ARC by targeting silencing p53. In addition, other studies have shown that miR-31, miR-99a/b, and let-7b are differentially expressed in lens tissues of ARC [31]. In this study, we also found that miR-139-5p was abnormally expressed in lens of patients with cataract. Furthermore, we found that miR-139-5p could directly target the 3'UTR of caspase-3 and negatively regulate caspase-3 expression. Caspase-3 is a key protease involved in mammalian cell apoptosis [32]. Recently, studies found that the levels of caspase-3 and Bax in the lens of patients with cataract were increased and the Bcl-2 level was decreased [33], which was consistent with our results. Hence, we speculated that miR-139-5p might exert its function in ARC by regulating the expression of caspase-3. Further studies confirmed this hypothesis. In SRA01/04 cells treated with UV irradiation, overexpression caspase-3 could reverse the inhibitory effect of knockdown of CASC15 and miR-139-5p mimic on SRA01/04 cell apoptosis.

In conclusion, our data indicated that CASC15 could promote LECs apoptosis by regulating miR-139-5p/caspase-3, which provided a new strategy for the treatment of ARC.

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