

miR-211 regulates the antioxidant function of lens epithelial cells affected by age-related cataracts

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Received: 2017-08-10 Accepted: 2018-01-10

Abstract

• **AIM:** To investigate the effects and mechanism of miR-211 in mediating the antioxidant function of lens epithelial cells affected by age-related cataracts.

• **METHODS:** Real-time quantitative polymerase chain reaction (RT-qPCR) was used to detect miR-211 expression in the anterior lens capsules of healthy people, the anterior lens capsules of patients with age-related cataracts, and human epithelial cell line (SRA01/04) cells exposed to oxidative stress. A 2', 7'-dichloro-fluorescein diacetate (DCFH-DA) probe was used to measure the levels of endogenous reactive oxygen species (ROS) in human lens epithelial cells (hLECs) exposed to 400 $\mu\text{mol/L}$ H_2O_2 for 1h. SRA01/04 cells were transfected with either miR-211 mimics, mimic controls, miR-211 inhibitors or inhibitor controls. After 72h, these cells were exposed to 400 $\mu\text{mol/L}$ H_2O_2 for 1h, then p53 and Bax mRNA expression were measured using RT-qPCR. p53 and Bax protein expression were also measured by Western blotting analysis. Finally, cell viability was assessed using an MTS assay.

• **RESULTS:** Compared to the control group, expression of miR-211 in the anterior lens capsules of age-related cataract patients and in SRA01/04 cells exposed to oxidative stress was significantly increased ($P<0.001$). Levels of endogenous ROS were significantly elevated in hLECs exposed to oxidative stress ($P<0.001$). Compared to the mimic control group, the hLECs in the miR-211 mimic

group expressed significantly higher levels of p53 and Bax mRNA and protein while cell viability was significantly reduced ($P<0.001$). Conversely, p53 and Bax mRNA and protein expression were significantly reduced in the miR-211 inhibitor group as compared to the control group, while the cells in this group had much higher levels of cell viability ($P<0.001$).

• **CONCLUSION:** miR-211 is upregulated in the anterior lens capsules of age-related cataract patients. miR-211 decreased the antioxidative stress capacity of lens epithelial cells by upregulating p53 and Bax, while inhibiting cell proliferation and repair. This finding suggests that miR-211 may play a key role in the development of age-related cataracts.

• **KEYWORDS:** miR-211; p53; Bax; age-related cataract; oxidative stress

DOI:10.18240/ijo.2018.03.01

Citation: Lu B, Christensen IT, Ma LW, Yu T, Jiang LF, Wang CX, Feng L, Zhang JS, Yan QC, Wang XL. miR-211 regulates the antioxidant function of lens epithelial cells affected by age-related cataracts. *Int J Ophthalmol* 2018;11(3):349-353

INTRODUCTION

Cataract is a common age-related disease and its incidence continues to rise^[1]. Due to the unclear nature of its pathogenesis, non-surgical treatment of cataracts has remained limited. Previous studies of the pathogenesis of cataracts have provided evidence to suggest that oxidative damage may be a key factor in cataract development^[2-3]. One study found a significantly elevated level of reactive oxygen species (ROS) in aqueous humor and lens of patients with age-related cataracts^[4], while a separate *in vitro* study has demonstrated that hydrogen peroxide (H_2O_2) concentration equal to that in the lens of cataract patients may lead to lens opacity and lens epithelial cell apoptosis^[5].

MicroRNAs (miRNAs) are the short noncoding RNAs of 21-23 nucleotides in length, which can bind to the 3'-untranslated region (UTR) of target mRNAs, resulting in the translational repression or degradation of mRNA^[6]. Prior studies have shown that miRNAs are involved in a variety of physiological and pathological processes^[7]. It has also been shown that some miRNAs are associated with the onset of age-related cataracts, suggesting that miRNAs may become a new target for cataract diagnosis and treatment^[8].

miR-211 is located on intron 6 of the *Trpm1* gene at 15q13-q14, a locus that is frequently lost in many neoplasms^[9]. miR-211 is one of the most abundant miRNAs in the developing and adult eye^[10]. miR-211 belongs to a group of specific miRNAs that are highly expressed in human vitreous^[11].

Recent studies have found that miRNAs regulate human lens epithelial cell (hLEC) apoptosis^[8,12] and thus may be involved in the development of cataracts. Expression of miR-211 specifically has been found to play a key role in retinal pigment epithelium (RPE) cell differentiation and function^[13-14]. However, the precise relationship between miR-211 and oxidative damage leading to cataract formation has not been previously reported. Thus, in this study, we measured the expression of miR-211 in age-related cataract lens tissue, and then investigated the role and mechanism of miR-211 in the oxidative stress response and the process of age-related cataract formation.

SUBJECTS AND METHODS

Specimens A total of 21 fresh anterior lens capsules were collected between January 2016 and March 2016 at the Fourth Affiliated Hospital of China Medical University from age-related cataract patients undergoing phacoemulsification surgery (patients were excluded if they were affected by other eye diseases). Nine of the samples were collected from males and 12 from females, aged 56-72 (61.31±8.23)y. Fifteen transparent (healthy) anterior lens capsules were collected from the Fourth Affiliated Hospital of China Medical University Eye Bank, including 9 from males and 6 from females, aged 51-69 (60.24±7.32)y. All specimens were immediately stored in liquid nitrogen at the time of collection. This study was approved by the Ethics Committee of the Fourth Affiliated Hospital of China Medical University, and signed informed consent was obtained from each patient.

Cell Culture A human lens epithelial cell line (SRA01/04) was generously donated for experimental use by Dr. Yi-sin Liu of the Doheny Eye Institute. This cell line was cultured in a medium containing 10% fetal bovine serum (Gibco, USA), 100 U/mL penicillin and 100 mg/mL streptomycin (Thermo, USA) in DMEM medium (Invitrogen, USA), and was placed in a 37°C, 5% CO₂ constant temperature incubator.

Real-time Quantitative Polymerase Chain Reaction Trizol reagent (Invitrogen, USA) was used to extract total cell RNA. Then, a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, USA) was used to obtain miRNA cDNAs. TaqMan MicroRNA Assays (Applied Biosystems, expression USA) were used to detect miR-211 expression, and RNU6B was employed as an internal control. RNA was reverse transcribed using the PrimerScript RT reagent kit (Takara, China) and SIRT1 mRNA expression was detected using a TaqMan Universal Master Mix II (Applied Biosystems, USA), with β-actin designated as an internal control. The upstream and downstream primers of miR-211 and RNU6B were purchased from ThermoFisher (USA) and their sequences can be found

on their website. The p53 primer sequences are as follows: forward: 5'-CAGCAGTCAAGCACTGCCAAG-3', reverse: 5'-AGACAGGCATGGCACGGATAA-3'. The Bax primer sequences are as follows: forward: 5'-AGATGAACTGGACAGCAATATG-3', reverse: 5'-CCTACCCAGCCTCCGTTAT-3'. The β-actin primer sequences were: forward: 5'-CATCCGTAAGACCTCTATGCCAAC-3', reverse: 5'-ATGGAGCCACCGATCCACA-3'. PCR was performed using the ABI 7500 (Applied Biosystems, USA). Three independent experiments were performed and 2^{-ΔΔCt} quantitative analysis was performed to analyze relative expression levels.

Detection of Endogenous Intracellular Reactive Oxygen Species

A 2',7'-dichloro-fluorescein diacetate (DCFH-DA) probe was used to detect fluorescence derived from endogenous ROS in hLECs. Totally 1×10⁴ cells were seeded into each well of a 96-well plate and were cultured for 16h, until cells were observed adhering to the sides of the well. The cells were then exposed to 400 μmol/L H₂O₂ for 1h whereupon the culture medium was aspirated and 10 μmol/L fluorescent probe DCFH-DA was added to each well. This mixture was then incubated in a 37°C incubator for 20min. The cells were then washed three times with phosphate buffer solution and their DCF fluorescence intensity value (*i.e.* the mean fluorescence intensity of DCF, representing the level of intracellular ROS) was read using a multifunctional microplate reader. The excitation wavelength used was 485 nm and the emission wavelength was set at 530 nm.

Cell Transfection SRA01/04 cells were seeded in 24-well cell culture plate for 24h. Then Lipofectamine RNAiMAX Transfection Reagent (Invitrogen, USA) was used according to the manufacturer instructions to transfect the cells with miR-211 mimics, mimic controls, miR-211 inhibitors, or inhibitor controls. After 72h, cells were exposed to 400 μmol/L H₂O₂ for 1h whereupon mRNA and protein levels of p53 and Bax were measured using RT-qPCR and Western blotting. An MTS assay was used to assess cell viability.

Cell Viability Assay Cell viability was detected using the MTS assay kit (Promega, China). Seventy-two hours after transfection, SRA01/04 cells were exposed to 400 μmol/L H₂O₂ for 1h. According to the manufacturer's instructions, 20 μL MTS solution was added to each well and the cells were then incubated for 4h at 37°C, 5% CO₂. The absorbance of each group was read using an absorbance plate reader set to 490 nm wavelength.

Western Blotting Protein RIPA lysis buffer (Pierce, USA) was used to extract total protein and a BCA kit (Thermo, USA) was employed to quantify protein concentration. Forty microgramme of protein solution was added to each well of 10% NuPAGE Bis-Tris precast gels (Invitrogen, USA) for electrophoretic separation of proteins. Proteins were then transferred to PVDF membranes and blocked with 5% nonfat dry milk for 1h at room temperature. The protein was then incubated with primary antibody: rabbit anti-p53 (1:1000,

abcam, USA), rabbit anti-Bax (1:1000, abcam, USA) or rabbit anti-GAPDH (1:2000, abcam, USA) overnight at 4°C. HRP-labeled goat anti-rabbit IgG (H+L) secondary antibody (1:2500, Promega, USA) was then added and samples were incubated at room temperature for 2h. Proteins were then detected using an ECL kit after which analysis of protein bands was conducted using Image J software (USA).

Statistical Analysis Each experiment was performed independently at least 3 times with similar results. Measurement data were presented as mean±standard deviation (SD). Differences between the groups were calculated using unpaired Student's *t*-tests. Statistical significant difference was considered at $P<0.05$. Statistical analysis was done using SPSS 16.0 (USA).

RESULTS

miR-211 Expression Increased in the Anterior Lens Capsules of Patients with Age-related Cataracts RT-qPCR detection of the expression level of miR-211 indicated that, as compared to the healthy transparent lens capsule group (normal group), the level of miR-211 expression in anterior capsules of patients with age-related cataracts (cataract group) was significantly higher (4.23 ± 0.27 , $P<0.001$).

miR-211 Expression Increased in hLECs Subjected to Oxidative Stress Based on the results of the DCFH-DA fluorescent probe assay, endogenous ROS levels were significantly increased in SRA01/04 lens epithelial cells exposed to 400 $\mu\text{mol/L}$ H_2O_2 (H_2O_2 group, 264.64 ± 32), relative to the control group (53.35 ± 9.12 , $P<0.001$). miR-211 expression in the H_2O_2 group was also significantly higher than the control group as measured by RT-qPCR (5.24 ± 0.38 , $P<0.001$).

miR-211 Regulated the Oxidative Stress Response in hLECs Seventy-two hours after transfection, SRA01/04 cells were exposed to 400 $\mu\text{mol/L}$ H_2O_2 for 1h. Cell viability was detected using the MTS assay. It was found that, compared with the mimic control group, the cell viability of the miR-211 mimic group lens epithelial cells was significantly reduced ($P<0.001$; Figure 1). Compared to the control group, the cell viability of the lens epithelial cells in the miR-211 inhibitor group was significantly increased ($P<0.001$; Figure 1) suggesting that miR-211 suppresses proliferation in hLECs and diminishes their ability to cope with oxidative stress.

miR-211 Increased the Relative Expression of p53 and Bax mRNA in hLECs Using RT-qPCR, it was found that, compared with the mimic control group, the miR-211 mimic group hLECs had significantly increased levels of p53 and Bax mRNA expression ($P<0.001$; Figure 2). Conversely, p53 and Bax mRNA expression was significantly reduced in the miR-211 inhibitor group as compared to the inhibitor control group ($P<0.001$; Figure 2).

miR-211 Increased the Relative Expression of p53 and Bax Protein in hLECs By Western blotting, it was found that the miR-211 mimic group hLECs had significantly increased

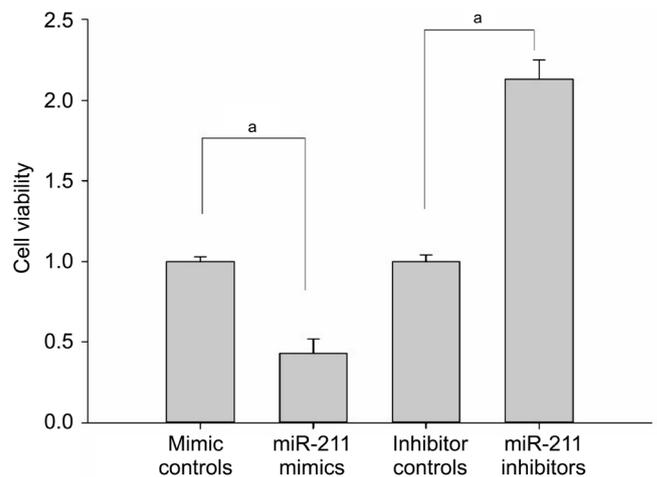


Figure 1 Cell viability of hLECs measured by MTS assay Seventy-two hours after transfection, SRA01/04 cells were exposed to 400 $\mu\text{mol/L}$ H_2O_2 for 1h. Cell viability was detected using the MTS assay ($^aP<0.001$).

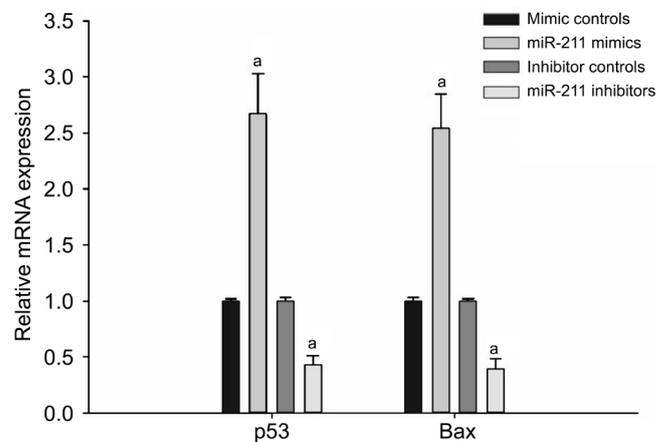


Figure 2 RT-qPCR detection of the expression of p53 and Bax mRNA in hLECs Compared with the mimic control group, the miR-211 mimic group hLECs had significantly increased levels of p53 and Bax mRNA expression. Conversely, p53 and Bax mRNA expression were significantly reduced in the miR-211 inhibitor group as compared to the inhibitor control group ($^aP<0.001$).

levels of p53 and Bax protein expression when compared with the mimic control group ($P<0.001$; Figure 3). Conversely, p53 and Bax protein expression were significantly reduced in the miR-211 inhibitor group as compared to the inhibitor control group ($P<0.001$; Figure 3).

DISCUSSION

As stated in the introduction, cataracts are one of the most common ocular diseases, and a leading cause of blindness worldwide^[15]. Current treatments of this condition are almost exclusively surgical, and cataract surgery is the most common surgical procedure performed on individuals over 65y^[16-17]. Surgical treatment, while effective, often incurs a large economic cost and generates a great deal of fear in patients^[18-19]. Therefore, investigation into effective non-surgical interventions for cataracts is an important endeavor with the potential for widespread application and great social benefit.

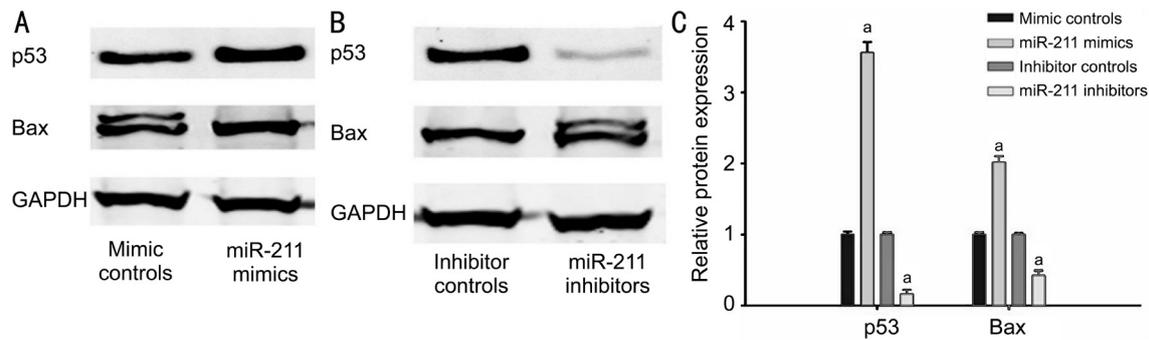


Figure 3 The expression of p53 and Bax protein in hLECs A, C: The miR-211 mimic group hLECs had significantly increased levels of p53 and Bax protein expression when compared with the mimic control group; B and C: p53 and Bax protein expression were significantly reduced in the miR-211 inhibitor group as compared to the inhibitor control group (^a $P < 0.001$).

Previous research has reported that a variety of stimuli have the potential for generating oxygen free radicals in the eye and that the resultant oxidative stress plays an important role in the pathogenesis of cataracts^[20-22]. Oxidative stress refers when to the exogenous or endogenous ROS exceeds the antioxidant capacity of a cell and exerts a deleterious impact on the cellular signal transduction system, and further damages nucleic acids, proteins, lipids and other macromolecules^[4,23-24]. Excess of ROS are often associated with pathology and have been implicated in causing cellular aging, apoptosis and necrosis^[20,25-26]. It has also been previously reported that hydrogen peroxide (H_2O_2) levels are significantly increased in the aqueous humor of cataract^[4]. A study by Spector and Garner^[27] showed that early cataract development is associated with an increase of oxidative stress. This study further demonstrated that H_2O_2 levels in cataract patients' aqueous humor increased as much as 30 times higher than normal. *In vitro* studies have demonstrated that equivalent levels of hydrogen peroxide can induce apoptosis in lens epithelial cells and generate lens opacity in a similar manner as that occurs in the eyes of cataract patients^[5]. However, the mechanism by which increased H_2O_2 causes such pathological changes in the lens remains unclear.

miRNAs are a class of endogenous noncoding RNA that often regulate gene expression by either complementary pairing with the 3'-UTR of a target gene or mediating the degradation of target gene mRNA^[28]. One miRNA may target more than one mRNA, and they are involved in a variety of physiological and pathological processes including cell growth and apoptosis, hormone secretion, aging, organ development, immune response and other pathologies^[7,29]. Although many miRNAs have been studied in relation to age-related cataract development, the relationship between miR-211 and oxidative damage in the development of cataracts has not been previously investigated. Previous research has reported that miR-211 expression is low in breast cancer, malignant melanoma, liver cancer, and a variety of other diseases such as vitiligo^[30-33]. Unrelated studies have also shown that miR-211 expression plays a key role in the differentiation and normal function of retinal pigment epithelium cells^[13-14].

This study found that miR-211 is highly expressed in age-related cataract tissue, suggesting that miR-211 may be involved in the incidence of age-related cataracts. miR-211 expression was also found to be increased in lens epithelial cells with H_2O_2 -induced oxidative stress, further suggesting that miR-211 is involved in the oxidative stress pathway of hLECs. The results of the subsequent experiments employing an H_2O_2 -induced oxidative stress model with miR-211 mimic transfection further established this relationship. The group of lens epithelial cells transfected with miR-211 mimics had a greatly decreased cell viability when compared to the control group while the lens epithelial cells transfected with miR-211 inhibitors had increased cell viability. In addition, p53 and Bax (key regulators of apoptosis) mRNA and protein expression levels displayed a direct relationship with miR-211 expression, suggesting that miR-211 may decrease lens epithelial cell viability and its ability to cope with oxidative stress by modulating p53 and Bax expression.

In summary, miR-211 expression is increased in age-related cataract tissues and miR-211 appears to participate in the development of this disease as it diminishes the ability of hLECs to defend against oxidative stress, and inhibits hLEC proliferation and repair by up-regulating p53 and Bax. The findings of this study provide strong evidence that miR-211 could be a potential new target for the diagnosis and treatment of cataracts.

ACKNOWLEDGEMENTS

Conflicts of Interest: Lu B, None; Christensen IT, None; Ma LW, None; Yu T, None; Jiang LF, None; Wang CX, None; Feng L, None; Zhang JS, None; Yan QC, None; Wang XL, None.

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