Association of alpha A-crystallin polymorphisms with susceptibility to nuclear age-related cataract in a Han Chinese population

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Abstract

**Background:** Alpha A-crystallin (CRYAA) is considered critical for the maintenance of lens transparency and is related to the pathogenesis of age-related cataracts (ARCs), especially the nuclear subtype. As the 5′ untranslated region (5′ UTR) modulates gene expression, the purpose of current study was to investigate whether single nucleotide polymorphisms (SNPs) in the 5′ UTR of CRYAA were associated with susceptibility to ARC in a Han Chinese population and to clarify the mechanism of this association.

**Methods:** SNPs in the 5′ UTR (−1 to −1000) of CRYAA were identified in 243 nuclear ARC patients and 263 controls using polymerase chain reaction and DNA sequencing. Allele and genotype frequencies were calculated and compared between two groups. Haploview 4.2 was used to calculate the linkage disequilibrium index, and the SHEsis analysis platform was used to infer haplotype construction. A dual-luciferase reporter gene assay was used for transcription of CRYAA in the presence of a protective haplotype with individual SNP alteration, Chromatin immunoprecipitation (ChIP) was employed to determine whether SNPs regulated CRYAA expression by altering the binding affinity of transcription factors.

**Results:** Three polymorphisms were identified in the 5′ UTR of CRYAA: rs3761381 (P = 0.000357, odds ratio [OR] = 1.837), rs13053109 (P = 0.788, OR = 1.086), and rs7278468 (P = 0.00136, OR = 0.652). The haplotype C-G-T (P = 0.0014, OR = 1.536) increased the risk of nuclear ARC, whereas the haplotype T-G-G (P = 0.00029, OR = 0.535) decreased the risk. The haplotype C-G-T decreased CRYAA transcription through rs7278468, which is located in the binding site of specificity protein 1 (Sp1). Furthermore, the G allele of rs7278468 increased CRYAA transcription by enhancing the binding affinity of Sp1.

**Conclusions:** These data indicate that the CRYAA polymorphism is a genetic marker of inter-individual differences in the risk of nuclear ARC.

**Keywords:** Alpha A-crystallin, Age-related cataract, 5′ untranslated region, Single nucleotide polymorphisms, Transcriptional activity
Background
Age-related cataracts (ARCs), which are a major cause of blindness worldwide, are characterized by lens opacities and visual impairment due to degenerative changes in the lens in the elderly [1]. The causes of ARCs are multifactorial, with both environmental and genetic variations implicated in the disease [2]. A study of twins strongly implicated genetic factors in the pathogenesis of ARCs, demonstrating heritability of 48% for the nuclear subtype [2]. Recent studies also indicated that several genes, such as galactokinase and eph-receptor tyrosine kinase-type A2 [3, 4], were genetic risk factors for cataracts. Although the importance of genetic risk factors for ARC has been highlighted, the pathophysiology is far from clearly understood.

As a major structural protein component expressed in the lens, alpha A-crystallin (CRYAA) is considered critical for the maintenance of lens transparency [5]. Many studies showed that CRYAA was related to the pathogenesis of ARC, including a study conducted by our research group [6–8]. The chaperone-like activity of CRYAA enables it to protect other crystallins against thermally induced inactivation or aggregation [9]. In addition, CRYAA can trap aggregation-prone denatured proteins, an action that is thought to delay the development of ARC [10]. Although previous research demonstrated that the levels of CRYAA decreased in the nuclear capsule of ARC patients compared to those of controls [8], the mechanism underlying the downregulation of CRYAA in the lens was unclear.

Previous research demonstrated that the 5′ untranslated region (5′ UTR) acted as a regulatory element in genes and that it was associated with modulating the expression of gene-coding regions [11]. Single nucleotide polymorphisms (SNPs) in 5′ UTR sequences were shown to play a critical role in regulating gene expression [12]. In addition, previous studies showed that SNPs in the 5′ UTR of the SLC16A12 gene were involved in the pathogenic expression of CRYAA [13]. Recently, SNPs that were identified in the CRYAA gene promoter region that were associated with cortical ARC in a Northern Italian population [14]. However, it is unknown whether SNPs in the 5′ UTR of CRYAA contribute to ARC susceptibility in a Han Chinese population, especially the nuclear subtype (the most frequent form).

The present study focuses on polymorphisms within the 5′ UTR of CRYAA (~1 to ~1000) and attempts to shed light on the development of nuclear ARC in a Han Chinese population.

Methods
Subjects
Subjects were recruited from the Eye and ENT Hospital of Fudan University. All the subjects underwent a full ophthalmic examination, including visual acuity, slit-lamp microscopic examinations, and ophthalmoscopic examinations. There was no consanguinity between the subjects (at least not among all four grandparents). All the enrolled subjects self-identified as Han Chinese (all four grandparents were ethnic Han Chinese). This research was approved by the Institutional Review Board and followed the tenets of the Declaration of Helsinki. All the subjects signed informed consent forms.

Lens opacity grading
After pupil dilation with 1% tropicamide, a trained ophthalmologist graded the lens opacity of each right eye according to the Lens Opacity Classification System (LOCS) III. Opacity was classified as nuclear opalescence (NO), nuclear color (NC), cortical (C), and posterior subcapsular (P). The grading consisted of six standards for NO and NC (standards 1 to 6) and five standards for C and P (standards 1 to 5). Each standard was assigned using decimals to interpolate between the reference standards, with the assigned scores ranging from 0.1 to 6.9 for NO and NC and 0.1 to 5.9 for C and P. The subtype of the cataract was then classified as nuclear (NO or NC ≥3), cortical (C ≥ 2), posterior subcapsular cataract (PSC) (P ≥ 2), or mixed type (i.e., the presence of more than one type in one eye).

ARC group and control group
All subjects with nuclear cataract were enrolled. Subjects with cortical and posterior subcapsular cataracts were excluded from this study. For the mixed type, only subjects with NO or NC scores higher than C and P scores were enrolled. Cases and controls were then recruited based on their NO and NC grading scores. The cases included subjects with NO and NC grading scores ≥3.0, and the controls included those with NO and NC grading scores <2.0.

The exclusion criteria for the cases and controls included: (1) subjects younger than 45 years; (2) pseudophakia or aphakia in either eye; (3) the presence of other eye diseases, such as dislocated lens, trauma, uveitis, high myopia, glaucoma, macular diseases, and retinal detachment; (4) previous ocular surgery in either eye; and (5) a history of diabetes, kidney disease, respiratory disease, cancer, or tumors.

Blood sample collection and DNA isolation
Five milliliters of peripheral blood samples were collected in EDTA tubes from all the subjects was and stored at −80 °C until use. DNA was isolated from whole blood cells using a Mammal Blood Genomic DNA Extraction Kit (LifeFeng Biotech Co., Shanghai, China), following the manufacturer’s protocol, and was stored at −20 °C until used for genotyping.
Identification and genotyping of SNPs
For analyzing the polymorphism in the 5′ UTR (−1 to −1000) of CRYAA, the polymerase chain reaction (PCR) and DNA direct sequencing were performed. A set of primers (forward: GGTGACACAGCAAGACTCCA and reverse: CACGTCCATGTTCAAGCTTTG) from Generay Biotechnology Co., Ltd. (Shanghai, China) was used to amplify the target fragment. The PCR reactions were performed in 50 μl reaction mixtures, consisting of 25 μl of PrimeSTAR Max Premix (Takara), 20 μl of RNase-free water, 1 μl of the forward primer, 1 μl of the reverse primer, and 1 μl of extracted genomic DNA. The PCR program included a 94 °C activation step for 3 min, followed by 30 cycles of 94 °C for 20 s, 60 °C for 20 s, 72 °C for 40 s, and 72 °C for 65 s. The PCR products were sequenced using ABI 3730xl (Generay Biotechnology Co., Ltd).

Cell culture and transfection
The human lens epithelium (HLE) B3 cell line obtained from American Type Culture Collection (ATCC; Rockville, MD, USA) was cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 20% fetal bovine serum (FBS). Before transfection, the cells were seeded in six-well plates and grown overnight, so that the cell density reached approximately 70%. A mixture of 2 μl of plasmid DNA and 4 μl of Lipofectamine™ 2000 (Invitrogen) reagent in 2 ml of serum-free medium was then added in a six-well plate. The cells were collected for luciferase activity testing and chromatin immunoprecipitation (ChIP) analysis 72 h and 48 h later, respectively.

Plasmid construction
The human CRYAA 5′ UTR (−1 to −1000) was amplified using primers, as described above and then inserted into the PGL3-Basic vector (Invitrogen) using the restriction enzymes Hind III and Kpn I (Takara). The CRYAA 5′ UTR containing each SNP mutation was constructed using the DLR™ luciferase activities were checked by a Dual-luciferase Reporter (Promega), according to the manufacture's protocol.

Luciferase assay
The HLE cells were cultured in six-well plates with 2 μg of either PGL3-Basic-CRYAA 5′ UTR or PGL3-Basic plasmid and 20 ng of pRL-TK and then transfected into cells, as described above. Then, 72 h after transfection, luciferase activities were checked by a Dual-luciferase Reporter (DLR™) Assay System (Promega), according to the manufacture's protocol.

In silico analysis
The transcription factor binding sites of the identified SNPs of CRYAA 5′ UTR were predicted by MEME SUITE as described previously [15].

Chromatin immunoprecipitation (ChIP) analysis
ChIP analysis of HLE cells transfected with a plasmid containing the CRYAA 5′ UTR was conducted 48 h after transfection using a Pierce Agarose ChIP Kit (Thermo Scientific), according to the manufacture's protocol. The antibodies used for ChIP included anti-human IgG (Abcam) and anti-specificity protein 1 (Sp1) (Abcam). The captured genomic DNA fragments were then purified with Premix Taq™ (Takara). The primers used for the ChIP PCR were as follows: CRYAA ChIP forward: CTGAG GACGTGTGACACTG, reverse: AGGCCTGGA CTCAGCTGA. CRYAA ChIP NC: forward: ACCCTGA CAGGAGCAGCCCC, reverse: TTCTCCAGGGTATC CAGTG. The ChIP-quantitative PCR (qPCR) data relative to Sp1 were calculated using the 2^−ΔΔCt methods and presented as % input.

Statistical analysis
Differences between the values were evaluated using a two-tailed Student's t-test or Fisher's chi-squared test, depending on the variables types. SPSS for Windows, version 17.0 (SPSS, IBM Inc., Chicago, IL, USA) was used in the statistical analysis of the above data and for the calculation of odds ratios (ORs). The Hardy–Weinberg equilibrium (HWE), linkage disequilibrium (LD) and the haplotype analysis was conducted using the SHesis software platform [16]. The experiments were all repeated at least three times. A value of P < 0.05 was considered statistically significant.

Results
Participant characteristics
In total, 243 unrelated ARC patients and 263 control subjects were included in this study. The mean ages of the ARC patients and controls were 70.06 ± 6.22 years and 69.01 ± 4.64 years, respectively (p = 0.667).

The SNPs identified in the CRYAA 5′ UTR
Three polymorphisms were identified in the 5′ UTR of CRYAA: rs3761381 (C > T), rs13053109 (G > C), and rs7278468 (T > G) (Fig. 1). The allele and genotype distributions of each identified SNP are shown in Table 1 and Table 2, respectively. All three SNPs were in Hardy Weinberg equilibrium (P > 0.05). The frequencies of the rs3761381 T allele (P = 0.000357, OR = 1.837, 95% confidence interval [95% CI] = 1.312–2.572) and rs7278468 G allele (P = 0.00136, OR = 0.847, 95% CI = 0.501–0.847) were significantly higher in the control group compared to the ARC group. With regard to rs13053109, there
were no significant differences in the genotype or allele frequency between the ARC patients and the controls.

**LD analysis and haplotype construction**

The D’ values for the LD of the three SNPs are shown in Fig. 2 (D’ values: all >0.9). As seen, there were very strong levels of LD in all three SNPs. Five possible haplotypes were constructed (Table 3). The haplotype C-G-T appeared to confer a high risk of ARC (\( P = 0.0014, \) OR = 1.536, 95% CI = 1.180–1.997), whereas the haplotype T-G-G (\( P = 0.00029, \) OR = 0.535, 95% CI = 0.381–0.753) seemed to confer protection against ARCs.

**The transcriptional activity of CRYAA in the presence of the haplotypes**

Based on the position of these three SNPs, we constructed luciferase reporter vectors containing the CRYAA 5’ UTR and either T-G-G (protective haplotype) or C-G-T (risk haplotype) and evaluated whether they could influence the transcriptional activity of the gene. Seventy-two hours after transfection of the HLE cells, a dual-luciferase reporter assay showed that the luciferase activity of the cells transfected with the vector containing the CRYAA 5’ UTR (Fig. 3) was more than 11 times higher than that of the cells transfected with the vector alone, demonstrating that the CRYAA 5’ UTR exhibited positive transcriptional activity in the HLE cells. The transcriptional activity of the CRYAA 5’ UTR with the risk-associated haplotype C-G-T was approximately 17% lower than that of the protective T-G-G haplotype (Fig. 3a). This finding indicated that the C-G-T haplotype reduced CRYAA transcriptional activity in HLE cells and that this haplotype might cause nuclear ARC by decreasing the transcriptional efficiency of CRYAA.

**The transcriptional activity of CRYAA in the presence of the protective haplotype with individual SNP alteration**

To distinguish which individual SNPs of the haplotypes in the CRYAA 5’ UTR were predominantly responsible for the regulation of transcriptional activity, site-directed mutagenesis was employed to alter the single base of each individual SNP present in the protective T-G-G haplotype, one by one. Subsequent transcriptional activity was then analyzed. As shown by a dual-luciferase reporter assay, 72 h after transfection, the transcriptional activities of the 5’ UTR containing the individual alleles of rs3761381 and rs13053109 occurring as a single base variation were not significantly different from those of

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**Table 1** Allele frequencies of SNPs in the CRYAA gene among the ARC group and the control group

| SNPs        | Allele | ARC group n (%) | Control group n (%) | P value | OR for minor allele (95% CI) |
|-------------|--------|-----------------|---------------------|---------|-----------------------------|
| rs3761381   | C      | 423 (87.0%)     | 413 (78.5%)         | 0.000357| 1.837 (1.312–2.572)         |
|             | T      | 63 (13.0%)      | 113 (21.5%)         |         |                             |
| rs13053109  | G      | 464 (95.5%)     | 504 (95.8%)         | 0.788   | 1.086 (0.594–1.988)         |
|             | C      | 22 (4.5%)       | 22 (4.2%)           |         |                             |
| rs7278468   | T      | 344 (70.8%)     | 322 (61.2%)         | 0.00136 | 0.652 (0.501–0.847)         |
|             | G      | 142 (29.2%)     | 204 (38.8%)         |         |                             |

SNPs: Single Nucleotide Polymorphisms, ARC: Age-Related Cataract, OR: Odds Ratio, CI: Confidence Interval
the protective T-G-G haplotype (Fig. 3b). However, when the G allele of rs7278468 replaced with the T allele, the consequent transcriptional activity of the CRYAA 5′ UTR with the T-C-T haplotype decreased approximately 11% (P < 0.05) compared with that of the protective T-G-G haplotype. These data indicated that the allelic change in rs7278468 affected CRYAA expression.

Table 2 Genotype frequencies of SNPs in the CRYAA gene among the ARC group and the control group

| SNPs      | Genotype | ARC group n (%) | Control group n (%) | P value |
|-----------|----------|-----------------|---------------------|---------|
| rs3761381 | C C      | 182 (74.9%)     | 161 (61.2%)         | 0.0011  |
|           | C T      | 59 (24.3%)      | 91 (34.6%)          |         |
|           | T T      | 2 (0.8%)        | 11 (4.2%)           |         |
| rs13053109| G G      | 222 (91.4%)     | 242 (92.0%)         | 0.965   |
|           | G C      | 20 (8.2%)       | 20 (7.6%)           |         |
|           | C C      | 1 (0.4%)        | 1 (0.4%)            |         |
| rs7278468 | T T      | 120 (49.4%)     | 99 (37.6%)          | 0.0054  |
|           | T G      | 104 (42.8%)     | 124 (47.1%)         |         |
|           | G G      | 19 (7.8%)       | 40 (15.2%)          |         |

SNPs Single Nucleotide Polymorphisms, ARC Age-Related Cataract

The rs7278468 G allele increased CRYAA transcription by enhancing the binding affinity of Sp1

Previous data showed that the rs7278468 alleles affected CRYAA transcriptional activity, as it lies upstream of the transcription start site. It is feasible that it might influence the binding affinity of one or more transcription factors and affect the transcriptional activity of CRYAA. Thus, using the MEME SUITE, transcription factor binding prediction of the sequence around rs7278468 was performed. The rs7278468 T allele is located in the binding motifs of several transcription factors, including specificity protein 1 (Sp1) (Additional file 1: Table S1). Sp1 is a transcriptional factor that applies its activity via binding to a GC-rich element in the promoter region of target genes. The methylation within the CpG sites of the CRYAA promoter might decrease the DNA-binding capacity of Sp1, leading to epigenetic repression in nuclear ARC lenses. The aforementioned factors make Sp1 a good candidate for further studies. To determine whether rs7278468 regulated CRYAA by altering the binding affinity of Sp1, ChIP-PCR was used to analyze the binding affinity of Sp1 in HLE cells (Fig. 4). With the ChIP-NC primers, bands were detected only in the input lanes and not in any of the immunoprecipitated samples. As shown by the ChIP-PCR, bands were detected in both the HLE cells alone and in the HLE cells transfected with the CRYAA_T-G-T or CRYAA_T-G-G plasmid. However, a higher strength ChIP-PCR band was detected in the CRYAA_T-G-G transfected cells (Fig. 4b), and the ChIP-qPCR confirmed this tendency (Fig. 4c). These data showed that Sp1 binds directly to the CRYAA 5′ UTR and that the rs7278468 G allele increased this binding. These findings implied that Sp1 might regulate the transcription of CRYAA by direct interaction with the 5′ UTR and that the rs7278468 G allele increased this interaction and enhanced transcriptional activity.

Discussion

This study demonstrated evidence of the involvement of alleles, genotypes, and haplotypes of SNPs in the 5′ UTR of CRYAA in the susceptibility to nuclear ARC. Similar results were reported previously in the case of all types of ARC (especially the cortical subtype) in a northern Italian population [14]. However, in the Han Chinese population with nuclear ARC in the current study, the sites of the SNP (rs3761381) and allelic frequency (rs7278468) were significantly different than those reported. In the present study, the frequency of the T allele of rs7278468 was higher (70.8%) than that (49.5%) reported in a previous study of patients with ARC. Numerous studies have demonstrated a population-specific and subtype-specific effect of SNPs, and ARC is no exception [17–19]. The increased frequency of the T allele of
rs7278468 observed in the present study might indicate an increased risk of the progression of ARC in the Han Chinese population, which was similar to epidemiological findings that the incidence rate of nuclear cataract in the Chinese population is higher than that in the Caucasian population [20, 21]. Furthermore, as rs7278468 has been found to be associated with cataracts in different populations, which points to the importance of the rs7278468 locus in the pathogenesis of cataracts, providing a potential target for future gene therapy. Additionally, the difference of allelic frequency between ours and Ma's study might be caused by selection bias. They included all types of ARC, but our study included only nuclear ARC. Therefore, further studies with all types of ARC should be considered.

As a molecular chaperone, CRYAA protects other crystallins from aggregation or inactivation and to traps aggregation-prone denatured proteins, which are suggested to delay the progression of ARC [10]. Studies with CRYAA-knockout animals and our previous reports demonstrated the importance of CRYAA in lens clarity [7, 22]. In a previous study, we showed that CRYAA expression decreased in the lens capsules of individuals with age-related nuclear cataracts compared to age-matched controls and confirmed lower CRYAA levels in samples with greater lens opacity severity [8]. The decrease in CRYAA expression may be one cause of lens opacity and also contributes to cataract pathology.

SNPs in the 5′ UTR play a critical role in gene expression and are involved in the pathophysiology of disease. Seshadri identified a SNP, rs366316, in the 5′ UTR of CD1a that was strongly associated with CD1a expression [23]. SNPs identified in the 5′ UTR of the SLC16A12 gene affected the pathogenic consequences of ARC [13]. As shown in the current study, Sp1 appeared to regulate CRYAA via binding to the binding motif in the 5′ UTR of CRYAA, with an increase in the binding strength of the Sp1 binding motif in the rs7278468 G allele increasing CRYAA transcription. The resulting increased in CRYAA transcription seemed to enhance the expression of the alpha A-crystallin protein in the lens, making individuals with the rs7278468 G allele invulnerable to the development of nuclear ARC.

Sp1 is a ubiquitously expressed transcription factor, which plays a critical role in regulating plenty of genes required for normal cell function [24, 25]. In human cells, SP1 acts as gene activator [25]. The DNA-binding affinity of Sp1 can be regulated both by altering protein interactions and by post-translational modification [26]. Recently, Liu reported that the methylation of CpG sites of the CRYAA promotor directly decreased the DNA-binding capacity of Sp1, leading to a reduction in the expression of CRYAA in HLE cells [27]. In the current study, Sp1 bound directly to the CRYAA 5′ UTR, and the rs7278468 G allele increased the binding affinity of Sp1, suggesting a mechanism for Sp1 regulation of CRYAA transcription.

### Table 3 Haplotype analysis of the ARC group and the control group

| Haplotypes | ARC group (freq) | Control group (freq) | P value | OR (95% CI) |
|------------|-----------------|----------------------|---------|-------------|
| C-C-G      | 22.00 (0.045)   | 22.00 (0.042)        | 0.788   | 1.087 (0.594–1.989) |
| C-G-G      | 59.31 (0.122)   | 71.32 (0.6136)       | 0.522   | 0.886 (0.613–1.282)  |
| C-G-T      | 341.69 (0.703)  | 319.68 (0.608)       | 0.0014  | 1.536 (1.180–1.997)  |
| T-G-G      | 60.69 (0.125)   | 110.68 (0.210)       | 0.00029 | 0.535 (0.381–0.753)  |
| T-G-T      | 2.31 (0.005)    | 2.32 (0.004)         | 0.695   | 1.247 (0.816–1.457)  |

ARC Age-Related Cataract, OR Odds Ratio CI Confidence Interval

**Fig. 3** Alleclic variation of the rs7278468 alters the transcription of CRYAA. a The luciferase activity of CRYAA 5′UTR within either the T-C-G or C-G-T haplotype after being transfected into HLE cells. b Site-directed mutagenesis was applied to discern the impact of rs3761381, rs13053109 and rs7278468. The allele rs7278468 is mainly accounted for the transcriptional activity alteration of the CRYAA 5′UTR. The CRYAA 5′UTR with T-C-G were set to 1, to which other values were normalized. The results are shown as the mean ± SD. (*p < 0.05)
To address the limitation of the sample size, larger sample studies are required to validate the association we have observed. Further studies could also be focused on the association of SNPs with cortical and PSC cataracts.

**Conclusions**

This study identified three SNPs and their haplotypes in the 5′ UTR of CRYAA that appeared to be associated with nuclear ARC. As shown by the analysis of the effects of the individual SNP alleles on CRYAA transcription, rs7278468 appeared to be mainly responsible for the alteration in the transcriptional activity of the 5′ UTR of CRYAA. It exerted this effect through its G allele, which strengthened the binding affinity of Sp1. Manipulating these polymorphisms may provide a strategy to prevent or slow the progression of ARC.

**Additional files**

**Additional file 1:** The transcription factor binding sites overlapping rs7278468. This is the raw data of the in-silico analysis. (DOCX 52 kb)

**Additional file 2:** Figures S1 and S2. The whole gels of the NC and Sp1 ChIPs respectively. This is the raw data of the chromatin immunoprecipitation (ChIP) analysis described above. (DOCX 645 kb)

**Abbreviations**

5′UTR: 5′ untranslated region; ARC: Age-related cataract; C: Cortical; ChIP: Chromatin immunoprecipitation; CI: Confidence interval; CRYAA: Alpha A-crystallin; DMEM: Dulbecco’s Modified Eagle Medium; FBS: Fetal bovine serum; HLE: Human lens epithelium; HWE: Hardy–Weinberg equilibrium; LD: Linkage disequilibrium; LOCS: Lens Opacity Classification System; NC: Nuclear color; NO: Nuclear opalescence; OR: Odds ratio; PCR: Polymerase chain reaction; PSC: Posterior subcapsular cataract; SNPs: Single nucleotide polymorphisms; Sp1: Specificity protein 1

**Funding**

This work was supported by the National Natural Science Foundation of China Grant (No. 81270989 and No. 81200669).

**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Authors’ contributions**

ZNZ carried out the Luciferase assay and ChIP analysis and drafted the manuscript. QF collected the blood sample and graded the lens opacity and participated in data collection. PZ and YL designed this study and helped in revising the manuscript. LC and HFY participated in design this study and analyzed the data. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

This research was approved by the Institutional Review Board the Eye and ENT Hospital of Fudan University and followed the tenets of the Declaration of Helsinki. All the subjects signed informed consent forms.

**Consent for publication**

Not applicable. This research did not involve individual participants.

**Competing interests**

The authors declare that they have no competing interests.

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