

# ARMS2 interference leads to decrease of proinflammatory mediators

Fanxing Zeng · Min Zhang · Yiting Xu · Haifeng Xu

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## Abstract

**Background** Age-related macular degeneration (AMD) is a major cause of irreversible blindness among elderly people in developed countries. Many studies suggested that age-related maculopathy susceptibility 2 (ARMS2) is the second major susceptibility gene for AMD. Increasing evidence was found recently that inflammatory processes and oxidative stress may contribute to the pathogenesis of AMD. Meanwhile, the mechanisms underlying the contributions of ARMS2 to the pathogenesis of AMD remain unclear. The purpose of the current study was to elucidate the relationship between the ARMS2 gene and proinflammatory mediators, for further assessment of the associated biologic effects.

**Methods** siRNA was used to knock down ARMS2 mRNA, and Western blotting and reverse real-time PCR were used to detect the effect of siRNA on the expression of ARMS2 in ARPE-19 cells. The expressions of C3, C5, IL-6, IL-8, and TNF- $\alpha$  after si-RNA knockdown were evaluated by SYBR Green I real-time PCR and ELISA.

**Results** Transcription accumulative indexes ( $TAI=2^{-\Delta\Delta CT}$ ) of ARMS2 by real-time PCR revealed that the transfection rate in the positive control group was  $72.0\pm 2.07\%$  ( $P<0.01$ ). The ratio of absorbance values (by Western blotting) of ARMS2 to  $\beta$ -actin was  $0.85\pm 0.122$ ,  $0.87\pm 0.143$ , and  $0.61\pm 0.240$  in the blank control group, scrambled ARMS2-siRNA group, and ARMS2-siRNA group respectively ( $F=42.5$ ,  $P<0.01$ ). The secreted protein levels of C3, C5, IL-6, IL-8, and TNF- $\alpha$  were found by ELISA to be reduced by  $34.24\pm 1.81\%$ ,  $37.15\pm 2.02\%$ ,  $35.11\pm 1.75\%$ ,  $30.11\pm 2.19\%$ , and  $34.33\pm 2.18\%$  respectively, in the siRNA-ARMS2 group

( $P<0.05$ ). Compared with the blank control group, reduced TAI of C3, C5, IL-6, IL-8, and TNF- $\alpha$  were detected by real-time PCR in the ARMS2-siRNA group.

**Conclusion** This study produced evidence supporting the notion that the ARMS2 risk allele for AMD is linked directly or indirectly to proinflammatory mediators. More importantly, our data indicate that the change in ARMS2 may affect C3, C5, IL-6, IL-8, and TNF- $\alpha$  levels, and this may be one of the mechanisms of AMD development.

**Keywords** Age-related maculopathy susceptibility 2 · Age-related macular degeneration · Retinal pigment epithelium

## Introduction

Age-related macular degeneration (AMD), a major cause of irreversible blindness among elderly people in developed countries, is a multifactorial disease with heterogeneous clinical manifestations [1]. In Asian populations, most vision-threatening cases of AMD are of the exudative type, while the atrophic type is more common in Caucasian populations [2, 3]. The cause of AMD is complicated because multiple genetic and environmental factors are involved in its pathogenesis. Epidemiological studies have indicated that factors such as age, smoking, gender, obesity, hypertension, and genetic background are associated with AMD [2, 4]. Increasing evidence has been found recently that inflammatory processes and oxidative stress may contribute to the pathogenesis of AMD [5–7].

Investigations on genome-wide and targeted genetic association have identified various polymorphisms that are important for AMD susceptibility, among which polymorphisms of complement factor H (CFH; Y402H) and age-related maculopathy susceptibility 2 (ARMS2; A69S) might have

F. Zeng · M. Zhang · Y. Xu · H. Xu (✉)  
Qingdao Eye Hospital, Shandong Eye Institute, Shandong Academy of Medical Sciences, 5 Yanerdao Road, Qingdao 266071, China  
e-mail: chxhf@126.com

the strongest influence on the pathogenesis of AMD in the Caucasian and Asian populations respectively [8–12]. The complement pathway is interfered with AMD by inflammatory process, but how ARMS2 influences the disease is still in dispute [6–8, 13–17]. Yasuma et al. [15] reported that people over 60 years carried AMD risk alleles in the ARMS2 locus; the serum high sensitivity C-reaction protein levels were elevated. They proposed that ARMS2 may heighten the risk of developing AMD by chronic systemic inflammation. The purpose of the current study was to elucidate the relationship between the ARMS2 gene and those proinflammatory mediators, so as to further assess the associated biologic effects.

## Materials and methods

### Cell cultures

Human ARPE-19 (ATCC, catalog No. CRL-2302, Rockefeller, Maryland, USA) cells were cultured in Dulbecco's modified Eagle medium/nutrient mixture F12 (DMEM/F12; Invitrogen, Carlsbad, CA, USA) (1:1) containing 10 % fetal bovine serum (FBS, Invitrogen), 100 IU/mL penicillin G (InvitrogenUSA), and 100 µg/mL streptomycin (Invitrogen). The cells were incubated at 37°C in a humidified atmosphere of 5 % CO<sub>2</sub> and 95 % air. The culture medium was changed every 3 days. After 90 % confluence was reached, the cells were routinely passaged by dissociation in 0.05 % trypsin/0.02 % ethylene diamine tetraacetic acid (EDTA). The cells were seeded in 96- or 6-well plates, according to the different requirements.

### Transfection of siRNA

The transfection reagent (FlexTube siRNA Premix) was purchased from Qiagen (Hilden, Germany), including a short-interfering RNA (siRNA) designed for the human ARMS2 gene (cat. no. SI00507990, target sequence CTCCATGATCC-CAGCTGCTAA) and a control siRNA, which contained a scrambled sequence (cat. no. SI03650318, target sequence CAGCATCAATGTGAAGCCAAA). FlexTube siRNA Premix was resuspended before the first use according to the manufacturer's protocol. Twenty-four hours before siRNA transfection, 250,000 cells/well were seeded in 6-well plates. At the time of transfection with siRNA, the cells were approximately 50–60 % confluent in 2 ml of complete DMEM/F12 medium, and then were transfected for 24 hrs with 42 µl of transfection reagent diluted in 2 ml of DMEM/F12 (HyClone, Thermo Fisher Scientific, Beijing, China) medium. The final concentration of the siRNAs was 25 nmol/l each. After transfection, the supernatant was removed from all wells, and some of the cells were incubated with complete DMEM/F12 medium for another 24 hrs to monitor changes in ARMS2 expression with real-time PCR, Western blotting, and ELISA.

Human ARPE-19 cells transfected with ARMS2-siRNA served as a positive control group to detect the transfection efficiency. The non-transfected ARPE-19 cells and scrambled transfected ARPE-19 cells with PBS and scrambled-ARMS2-siRNA were taken respectively as blank control and blank transfection groups. The forward and downward specific sequences for ARMS2, complement component 3 (C3), complement component (C5), interleukin (IL)-6, IL-8, tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), and  $\beta$ -actin are shown in Table 1.

Detection of ARMS2, C3, C5, IL-6, IL-8, and TNF- $\alpha$  by SYBR Green I Real-Time PCR

Total mRNA was extracted from ARPE-19 cells using a NucleoSpin RNAII Assay Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. The DNA concentration was standardized for working DNA using an Eppendorff pipetting machine (Eppendorf AG, Hamburg, Germany). The first strand complementary DNA (cDNA) was synthesized from 1 µg of total RNA in a 10-µl reaction mixture using a PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa, Dalian, China). PCR reactions were performed using GoTaq Flexi DNA polymerase (Promega, Madison, WI, USA) in a 10-µl mixture containing 0.25 µmol/L each of dNTP (Deoxyribonucleoside5'-Triphosphates), 2 µl 5× Colorless GoTaq Flexi Buffer, 2.5 mmol/l MgCl<sub>2</sub>, 0.25 mol/l of each primer, 4 units GoTaq DNA polymerase, and 25 ng cDNA. After an initial denaturation step at 95 °C for 3 min, amplification was performed for a total of 30 cycles under the following conditions: denature at 95 °C for 30 s, annealing at 40.7 °C for 30 s, and extension at 72 °C for 30 s. Then the final step was at 72 °C for 10 min. A blank control substitution of water for the cDNA template was included in all experiments. The housekeeping gene  $\beta$ -actin was used as an internal control because it was believed to be continuously expressed in

**Table 1** Sequence of primers used for the real-time PCR

Target	Sequence of primer
ARMS2	forward-5'-TCG GTG GTT CCT GTG TCC TTC ATT-3' reverse-5'-TCA CCT TGC TGC AGT GTG GAT GAT-3'
TNF- $\alpha$	forward-5'- GTG ACA AGC CTG TAG CCC ATG TTA -3' reverse-5'- TTA TCT CTC AGC TCC ACG CCA TTG -3'
C3	forward-5'- AGG CCT CAG GAT GCC AAG AAT-3' reverse-5'- GAG CAA AGC CAG TCA TCA TGG-3'
C5	forward-5'- CCT GTT ACC AGT AAG CAA GCC AGA-3' reverse-5'- GAA TCA GGT AGG GCA AAC TGC AAT-3'
IL-6	forward-5'-AAG CCA GAG CTG TGC AGA TGA GTA-3' reverse-5'- TGT CCT GCA GCC ACT GGT TCA-3'
IL-8	forward-5'- GGC ACA AAC TTT CAG AGA CAG CAG-3' reverse-5'-GGC CAG CTT GGA AGT CAT GTT TAG-3'
$\beta$ -actin	forward-5'-ACC AAC TGG GAC GAC ATG GAG AAA-3' reverse-5'-ACT CCT GCT TGC TGA TCC ACA TCT-3'

cells. The purity and amount of RNA was determined using an ultraviolet (UV) spectrophotometer at 260 nm and 280 nm ( $A_{260}/A_{280} > 1.8$ ).

SYBR green I was diluted with a ratio of 1:10 000 and added to the reaction. The primers were designed with Primer 5.0. The mRNA expression was expressed as the transcription accumulative index ( $TAI = 2^{-\Delta\Delta CT}$ ). The three groups were used to detect the expressions of ARMS2, C3, C5, IL-6, IL-8, and TNF- $\alpha$ .

#### Western blotting assay

The cells in the three groups were seeded in tissue culture dishes, and allowed to grow for 72 hrs in the medium described above, before they were incubated for another 24 hrs in the same medium but without serum. Cells were lysed with lysis buffer containing 150 mM NaCl, 1 % Igepal, 1 mM EDTA, 50 mM Tris, and 10 % protease inhibitor mixture on ice for 30 min. The supernatants were extracted by centrifugation at 4 °C with 14,000 r/min and stored in -80 °C. Protein quantification was performed with the bicinchoninic acid (BCA) method according to the manufacturer's instructions (Thermo Scientific, Rockford, IL, USA). Thirty  $\mu$ g protein for each sample was subjected to 10 % SDS-PAGE, electrotransferred to nitrocellulose membrane (NC), blocked with 1 % Tris-Buffered Saline and Tween 20 (TBST)/5 % skimmed milk powder, incubated with ARMS2 (1:1000; Abcam, Cambridge, UK) primary antibody or  $\beta$ -actin (1: 20,000; Oncogene Research Products, San Diego, CA, USA) diluted in 1 % TBST/5 % skimmed milk powder overnight, rinsed three times in 0.1 % TBST, and incubated with Horseradish peroxidase labeled goat anti-rabbit IgG (1:5000, ZSGB-BIO, Beijing, China) for 2 hrs. After diaminobenzidin (DAB) coloration for 5–10 min, brown bands were taken as positive results. By using GAS9500 image analysis system (UVItecy Co., London, UK), the absorbance values of hybridity bands on NC membrane and the ratio to  $\beta$ -actin representing the protein relative quantity were determined.

#### Determination of C3, C5, IL-6, IL-8, and TNF- $\alpha$ by ELISA

The supernatants in the three groups were collected from cell cultures described above. The supernatants were tested by ELISA for C3, C5, IL-6, IL-8, and TNF- $\alpha$ . ELISA kits were purchased from R&D Systems, Inc. (Minneapolis, MN, USA) and were performed based on the kit instruction. Values were expressed as pg/ml of conditioned medium.

#### Statistical analysis

Data were expressed as  $\bar{X} \pm s$ . All analyses for statistically significant differences were performed with the one-way ANOVA test.  $P < 0.01$  was considered significant. All data were

presented as means (SD) of three independent experiments, each performed in duplicate.

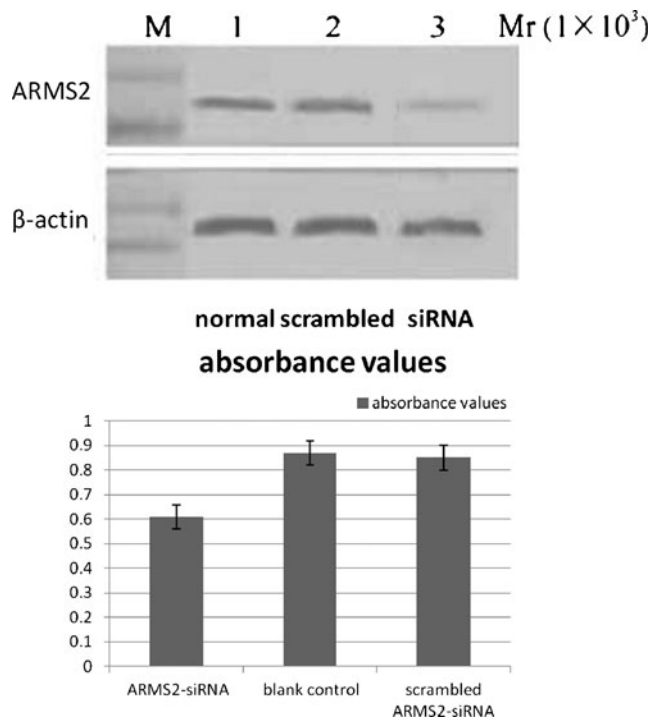
## Results

#### Transfection rate of siRNA SYBR Green I Real-Time PCR

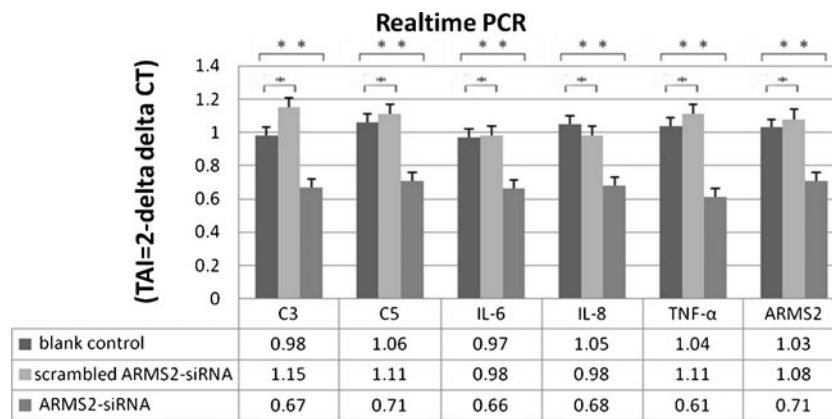
Transcription accumulative indexes ( $TAI = 2^{-\Delta\Delta CT}$ ) of ARMS2 in the blank control group, scrambled ARMS2-siRNA group, and ARMS2-siRNA group were  $1.06 \pm 0.043$ ,  $1.08 \pm 0.016$ , and  $0.71 \pm 0.011$  respectively ( $P < 0.01$ ) in an ABI 7500 real-time PCR (Applied Biosystems, Foster City, California, USA). The transfection rate in the positive control group was 72.0 % ( $\pm 1.07$ ,  $P < 0.01$ ).

#### Detection of ARMS2 protein expression by Western blotting

The ratio of absorbance values of ARMS2 to  $\beta$ -actin was  $0.61 \pm 0.240$ ,  $0.87 \pm 0.143$ , and  $0.85 \pm 0.122$  ( $F = 42.53$ ,  $P < 0.01$ ) (Fig. 1) in the ARMS2-siRNA group, blank control group, and scrambled ARMS2-siRNA group respectively. The ARMS2 level was decreased by  $29.0 \pm 1.12$  % after transfection in the ARMS2-siRNA group.



**Fig. 1** ARMS2 protein expression detected by Western blotting. *M*: marker; *1*: normal group; *2*: scrambled ARMS2-siRNA transfected group; *3*: ARMS2-siRNA transfected group. ARMS2 level was decreased by  $29.0 \pm 2.244$  % after transfection in the ARMS2-siRNA group. Significant difference was found between the ARMS2-siRNA group and the blank control group



**Fig. 2** Transcription accumulative indexes ( $TAI=2^{-\Delta\Delta CT}$ ) of ARMS2 in the blank control group, scrambled ARMS2-siRNA group, and ARMS2-siRNA group were  $1.06\pm 0.043$ ,  $1.08\pm 0.016$ , and  $0.71\pm 0.011$  respectively ( $P<0.01$ ), in an ABI 7500 real-time PCR (Applied Biosystems, Foster City, CA, USA). The transfection rate in the positive control group was 72.0 % ( $\pm 1.07$ ,  $P<0.01$ ). Compared with the blank control group, the TAI of C3, C5, IL-6, IL-8, and TNF- $\alpha$  in the ARMS2-siRNA group was reduced. In the normal group and the

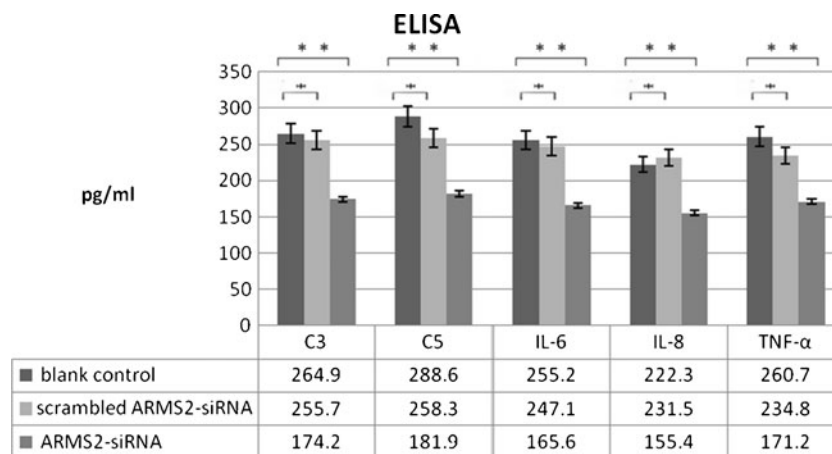
scrambled siRNA-transfected group, the expressions of C3, C5, IL-8, and TNF- $\alpha$  were similar, and no significant differences were found in these two groups ( $P=0.441$ ). No significant difference was found between the blank control group and the scrambled ARMS2-siRNA group (\*). Significant difference was found between the ARMS2-siRNA group and the blank control group (\*\*). Error bars indicate SD. \*\* $P<0.05$

Detection of C3, C5, IL-6, IL-8, and TNF- $\alpha$  expressions by real-time PCR

Transcription accumulative indexes ( $TAI=2^{-\Delta\Delta CT}$ ) of C3, C5, IL-6, IL-8, and TNF- $\alpha$  in differently treated cells are summarized in Fig. 2. Compared with the blank control group, the expressions of C3, C5, IL-6, IL-8, and TNF- $\alpha$  in the ARMS2-siRNA group was significantly reduced ( $P<0.05$ ). In the untreated and scrambled siRNA-transfected groups, the expressions of C3, C5, IL-6, IL-8, and TNF- $\alpha$  were similar, and no significant differences were found between these two groups ( $P=0.441$ ).

Detection of C3, C5, IL-6, IL-8, and TNF- $\alpha$  protein expressions by ELISA

The protein expression level was assayed by ELISA to examine the effect of siRNA-ARMS2 interference on C3, C5, IL-6, IL-8, and TNF- $\alpha$ . The values of C3, C5, IL-6, IL-8, and TNF- $\alpha$  in the three groups are shown in Fig. 3. The secreted protein levels of the siRNA-ARMS2 group continued to reduce by  $34.24\pm 1.812$  %,  $37.15\pm 2.021$  %,  $35.11\pm 1.751$  %,  $30.11\pm 2.191$  %, and  $34.33\pm 2.182$  % respectively ( $P<0.05$ ), after transduction as compared with the contents in the medium of the blank control group. No significant differences were



**Fig. 3** The secreted protein levels of the siRNA-ARMS2 group were reduced by  $34.24\pm 1.812$  %,  $37.15\pm 2.021$  %,  $35.11\pm 1.751$  %,  $30.11\pm 2.191$  %, and  $34.33\pm 2.182$  % respectively ( $P<0.05$ ), after transduction as compared with the content in the medium of blank control group. No

significant difference was found between the blank control group and the scrambled ARMS2-siRNA group (\*). Significant difference was found between the ARMS2-siRNA group and the blank control group (\*\*). Error bars indicate SD. \*\* $P<0.05$



found in the normal group and the scrambled siRNA-transfected group ( $P=0.343$ ).

## Discussion

Although it is generally accepted that both ARMS2 and inflammation are related with AMD, the association of ARMS2 and proinflammatory mediators has not been reported. In the current study, we investigated the effect of ARMS2 interference on the expression of some inflammatory cytokines. The results showed that interference of ARMS2 can down-regulate the expression of some of the major inflammatory cytokines, including IL-6, IL-8, and TNF- $\alpha$ , as well as the complement factors C3 and C5.

To date, the well documented alteration of inflammatory markers in AMD patients is the elevation of the C3, C5, CFH, IL-6, IL-8, and TNF- $\alpha$  levels [5–7]. These proinflammatory mediators are also associated with disease progression. CFH, C3, and C5 are main components of the alternative pathway. Factor H is an inhibitor of complement activation, while C3 is a central component of all complement activation pathways. Dysfunction of the complement system may result in local inflammation, autoimmune reactions, and tissue damage, and may play an important role in the etiology of AMD. IL-6 is a proinflammatory cytokine, which amplifies immune and inflammatory responses and plays a critical role in the occurrence of autoimmune diseases. It is also the key factor for the stimulation of immune reactions, inflammatory processes, and the occurrence of autoimmune diseases. Increased systemic or local IL-6 levels have been observed in various autoimmune diseases. IL-6-deficient mice were resistant to various experimentally-induced autoimmune diseases [13–17]. It has been reported that serum IL-6 levels correlated with the progression of AMD, and high levels of serum IL-6 were associated with the geographic atrophy type of AMD [13, 14]. IL-8 is a group of peptides produced by various types of cells, which activate and recruit polymorphonuclear leukocytes in acute and chronic inflammatory processes. It is supposed that IL-6 and IL-8 are released by degenerating retinal pigment epithelial cells and associated with drusen in AMD. TNF- $\alpha$  is a proinflammatory cytokine produced by macrophages and T-cells, and involved in inflammation and apoptosis. Activation of caspases, TUNEL-positive staining, and observation of hypodiploid cells suggest that apoptosis occur in cells treated with AMD alone or with AMD/TNF. In the eye, TNF- $\alpha$  appears to play a role in the pathogenesis of inflammatory, edematous, neovascular, and neurodegenerative disorders. Our results showed that all these inflammatory cytokines had a close relationship with the expression of ARMS2. A strong association between AMD and ARMS2 has been confirmed by multiple studies, but the exact mechanism remains unclear. The proposed functions of ARMS2 are in

mitochondria, extracellular matrix, or as a noncoding RNA [18–24]. Yasuma et al. [15] found that normal subjects with AMD risk alleles in the ARMS2/HTRA1 locus may be at risk of increased hypersensitive C-reaction protein levels and chronic systemic inflammation with aging. These findings indicate that the ARMS2 risk allele might increase the risk of AMD by inflammation pathway. Our study provided evidence supporting the above notion that the ARMS2 risk allele for AMD is linked directly or indirectly to chronic systemic inflammation.

Htra1 and ARMS2 are two neighboring genes located at chromosome 10q26. This area is regarded as a major candidate region associated with the susceptibility of several types of AMD [25], including polypoidal choroidal vasculopathy [26]. There is strong linkage disequilibrium across the ARMS2/HTRA1 region, making genetic association studies alone insufficient to distinguish the two candidates. Functional investigations of these two genes showed that decrease of ARMS2 message was associated with increase in HTRA1 expression [27–29]. HTRA1 is a multifunctional serine protease that is expressed in a variety of human tissues and cells, including the retinal pigment epithelium and vascular endothelium [24, 27–29]. Preliminary evidence suggests that Htra1 may increase the risk of AMD by inflammation pathway [24–29]. Therefore, we audaciously hypothesize that the ARMS2 gene might be involved in the inflammation mechanism through influencing Htra1 genes and subsequently the proinflammatory mediators.

There was an obvious limitation in this study. We did not investigate the relationship between ARMS2 and the protective factors, such as CFH, toll-like receptor-3 (TLR-3), IL-10, IL-27, etc. [30–32]. TLR-3 is an essential receptor of the innate immune system and the first responder for protection against bacterial and viral pathogens. Recently, genetic variants of both TLR3 and the key complement regulatory protein CFH were found to confer protection against AMD [32]. Hasegawa et al. [30] suggested that IL-10 had anti-angiogenic properties on choroidal neovascularization formation, and IL-27, a member of the IL-12 cytokine family, had an angiostatic effect and regulated the development of laser-induced experimental choroidal neovascularization in mice. IL-27 did not affect macrophage migration, but inhibited its VEGF production. However, our data indicated that ARMS2 was associated with the expression levels of C3, C5, IL-6, IL-8, and TNF- $\alpha$ . Our findings can further support the hypothesis that ARMS2 might be involved in the pathogenesis of AMD, at least partially, through inflammatory and immune-related mechanisms. However, the exact mechanism requires further investigations.

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**Conflict of interest** None.

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