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ORIGINAL RESEARCH

Associations of *ARMS2* and *CFH* Gene Polymorphisms with Neovascular Age-Related Macular Degeneration

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Supanji Supanji[1–4]
Dewi Fathin Romdhoniyyah[1,2]
Muhammad Bayu Sasongko[1,2,4]
Angela Nurini Agni[1,2,4]
Firman Setya Wardhana[1,2,4]
Tri Wahyu Widayanti[1,2,4]
Muhammad Eko Prayogo[1,2,4]
Ayudha Bahana Ilham Perdamaian[1,2]
Aninditta Dianratri[1,2]
Masashi Kawaichi[5]
Chio Oka[5]

[1]Department of Ophthalmology, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia; [2]Department of Ophthalmology, Dr. Sardjito General Hospital, Yogyakarta, Indonesia; [3]Ophthalmology Clinic, Military Air Force Central Hospital Dr. Suhardi Hardjolukito, Yogyakarta, Indonesia; [4]Ophthalmology Clinic, Dr YAP Eye Hospital, Yogyakarta, Indonesia; [5]Laboratory of Gene Function in Animals, Nara Institute of Science and Technology, Ikoma, Nara, Japan

Correspondence: Supanji Supanji
Department of Ophthalmology, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Jalan Farmako Sekip Utara, Yogyakarta, 55281, Indonesia
Tel +62 274 560300
Fax +62 274 581 876
Email supanji@ugm.ac.id

Purpose: This study aimed to determine the association of *ARMS2* A69S, *ARMS2* del443ins54, and *CFH* Y402H polymorphisms with neovascular age-related macular degeneration (nAMD) for the first time in an Indonesian population.

Patients and Methods: Our case–control study involved 104 nAMD and 100 control subjects. AMD diagnosis was evaluated by retinal specialists based on color fundus photography and optical coherence tomography. The polymorphisms on *CFH* Y402H and *ARMS2* A69S were analyzed by PCR-restriction fragment length polymorphism (PCR-RFLP), whereas *ARMS2* del443ins54 was evaluated by PCR-based assay.

Results: Significant allelic associations with nAMD were detected on all polymorphisms (P<0.05), with stronger association with the *ARMS2* A69S (OR 3.13; 95% CI 2.08–4.71; P<0.001) and *ARMS2* del443ins54 (OR 3.28; 95% CI 2.17–4.95; P<0.001) polymorphisms than with *CFH* Y402H (OR 2.08; 95% CI 1.08–3.99; P=0.028). Genotype analysis showed a statistical difference between nAMD and the control group for all polymorphisms (P<0.05). However, the association with nAMD was weaker for *CFH* Y402H (P=0.043) than for *ARMS2* A69S and *ARMS2* del443ins54 (P<0.001). A significant interaction between *ARMS2* A69S and hypertension was documented (OR 9.53; 95% CI 3.61–25.1; P<0.001).

Conclusion: Our findings indicate that *ARMS2* A69S and *ARMS2* del443ins54 polymorphisms are strongly associated with the risk of nAMD for the first time in an Indonesian population. The risk of nAMD increased when the presence of risk alleles from *ARMS2* A69S was combined with the presence of hypertension.

Keywords: age-related macular degeneration, *ARMS2*, *CFH*, polymorphism

Introduction

Age-related macular degeneration (AMD) is a progressive degenerative disease affecting the macula and is the top five leading cause of irreversible blindness worldwide.[1] It has been estimated that there are nearly 200 millions of individuals with AMD in 2020, and will be projected to rise to 288 millions in 2040.[1]

The prevalence of AMD increases exponentially with age.[1] With ageing, a cascade of deterioration occurs in photoreceptors, retinal pigment epithelium (RPE) and Bruch's membrane (BM) leaving permanent lesion observed clinically as geographic atrophy (dry AMD) or causing abnormal blood vessel originating from choroid to leak or to bleed at the macular area (neovascular AMD [nAMD]).[2] These may ultimately cause irreversible visual impairment if left untreated. Interestingly, studies showed that not all aged individuals undergo the similar

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processes and develop AMD, suggesting a strong genetic-driven variation in the pathophysiology of this condition.[3]

There has been extensive literature reporting the genetic associations in AMD.[4–6] Complement Factor H (CFH), Human high-temperature requirement serine protease A1 (*HtrA1*), and substitution from alanine to serine of amino acid 69 (A69S) in age-related maculopathy susceptibility 2 (*ARMS2*) at chromosome 10q26 are speculated to play key roles in cellular senescence, thus have been the most consistently associated with AMD in different populations.[7–9] In previous studies, *ARMS2* and *HtrA1* were reported to have a strong linkage disequilibrium.[10,11] Grassmann et al[12] further asserted that the *ARMS2* rs10490924 variant (not *HtrA1* rs11200638) is more strongly associated with AMD than *HtrA1* rs11200638. This finding was supported by Kanda et al,[10] who identify that *ARMS2* rs10490924 polymorphism alone can explain the association of the 200-kb region at chromosome 10q26 with AMD. Deletion/insertion consisting of a 443 bp deletion and an adjacent 54 bp insertion in the 3′-untranslated region (3′-UTR) of *ARMS2* (del443ins54) and complement factor H Tyr402His (*CFH* Y402H) was also reported to be strongly associated with AMD.[13–15] Deletion/insertion polymorphism in *ARMS2* disrupts the stability of *ARMS2* gene transcription products[16] and induces *HtrA1* transcription regulator activity.[17]

In Western populations, the associations of *ARMS2* and *CFH* were documented in American, Dutch, Italian, Spanish, and Swiss populations.[14,18–23] In Asian, similar associations were reported in Chinese, Japanese, and Indian populations.[15,24–27] However, very limited evidence is available from Asian Malay population, which is also one of the biggest ethnic groups in Asia.

In this study, we aimed to investigate the associations of *ARMS2* A69S, *ARMS2* del443ins54, and *CFH* Y402H with AMD in Indonesian population, which constitutes the majority of Asian Malay ethnic group in the region.

Method

This was an age-matched case–control study of participants aged 45 years old or older. Cases were naïve nAMD patients in at least one eye attending retinal clinic at three tertiary hospitals in Yogyakarta: 1) Dr. Sardjito General Hospital; 2) Hardjolukito Military Air Force Central Hospital, and 3) Dr. YAP Eye Hospital with no previous history of AMD treatment, recruited consecutively from August 2016 to November 2018. The diagnosis of AMD was established from slit-lamp examination, fundus photograph and spectral-domain OCT, confirmed by a retinal specialist following the International Age-related Maculopathy (ARM) Epidemiological Study Group[28] and AMD clinical classification criteria.[29] We excluded cases with co-existing choroidal or other retinal inflammatory diseases. Controls were healthy individuals without AMD or other retinal lesions who underwent eye examination for senile cataract.

Each subject was fully informed about the purpose and the procedures of the study. Consent was obtained from all subjects in written form prior to participation. All study procedures adhered to the principles of the Declaration of Helsinki. The study was approved by the Institutional Review Board of Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada in August 2016.

Genotyping

The genomic DNA of each patient was extracted from venous blood placed into a tube containing EDTA as an anticoagulant. The blood samples were immediately processed utilizing a commercially available DNA extraction kit (GeneAid Genomic Human DNA Mini Kit [GB100/300], New Taipei City, Taiwan). DNA extraction and single nucleotide polymorphism (SNP) identification were conducted at the Integrated Research Laboratory, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada.

The specific variants for the *ARMS2* genes were *ARMS2* A69S rs10490924 and *ARMS2* del443ins54 (c.*372_815del443ins54), whereas that for *CFH* Y402H was rs1061170. Polymerase chain reaction (PCR) was performed in a thermal cycler (ProFlex PCR System, Applied Biosystems) following the ready-to-use PCR kit protocol (KAPA Taq PCR Kit, Kapa Biosystems). The PCR cycling conditions were set as follows: 1 cycle (95 °C for 2 min), 30 cycles (95 °C for 30 s), 1 cycle (52 °C for 1 min for each gene), 1 cycle (72 °C for 1 min), and 1 cycle (72 °C for 5 min).

The primer sequences for the genes of interest are as follows: 1) *ARMS2* A69S forward 5′-TGTCACTGCATTCCCTCCTGTCAT-3′ and reverse 5′-AAGCTTCTTACCCTGACTTCCAGC-3′; 2) *ARMS2* del443ins54 forward 5′-TACCCAGGACCGATGGTAAC-3′ and reverse 5′-GAGGAAGGCTGAATTGCCTA-3′; and 3) *CFH* Y402H forward 5′-CTTTAGTTCGTCTTCAGTTATAC-3′ and reverse 5′-GTCATCTATGTTACTTAGAAAGT-3′.

SNP identification involved PCR-based assay for *ARMS2* del443ins54 and PCR-restriction fragment length

polymorphism (PCR-RFLP) for *ARMS2* A69S and *CFH* Y402H. Restriction digestion was performed at 37 °C for 18 h following the manufacturer's protocol using PvuII restriction enzyme for *ARMS2* A69S (Takara Bio, Japan) and Hsp92II for *CFH* Y402H (Promega). All amplified products were electrophoresed on 1.5% agarose gel containing FloroSafe DNA stain (1st Base Asia). Random sampling from each genotype in each SNP was conducted for genotype confirmation through Sanger DNA sequencing. Sequencing service was provided by 1st Base Asia, Singapore.

Statistical Analysis

Descriptive data were generated for all variables. Unpaired Student's *t*-test for numerical variables or Chi-squared test and Fisher exact test for categorical variables was performed to compare baseline characteristics between nAMD and control groups. Two-sided p-values were reported. We tested for deviation from the Hardy–Weinberg equilibrium (HWE) in both groups through the chi-square test with the "genhwcci" command in Stata.

Associations between SNP and other risk factors for susceptibility to nAMD were assessed using logistic regression models measured by odds ratio (OR) and 95% confidence interval (CI). In the multivariable logistic regression model, the likelihood ratio test was performed to fit the model. We pooled one risk allele and two risk alleles as one category (risk allele) in the interaction analysis. Interaction analysis was performed by introducing the interaction term in the same regression model. All analyses were carried out using Stata (version 15.1, StataCorp, College Station, TX, USA).

Results

There were 116 cases [46 males (44.2%) and 58 females (55.8%)] and 100 controls [45 males (45.0%) and 55 females (55.0%)] included in the final analysis. Baseline characteristics of the participants are presented in Table 1. The mean age of cases was 66.3 ± 8.8 years while control was 67.9 ± 7.7 years. Cases showed very similar characteristics to control except that having higher BMI (23.7 vs 22.0; P=0.002) and were more likely to have hypertension (46.2% vs 18.0%; P<0.001) than controls.

The allele/genotype distributions and odds ratio (OR) of each SNP are summarized in Table 2. Significant allelic associations with nAMD were detected on all SNPs (P<0.05). Compared to those having non-risk alleles, those with risk alleles of *ARMS2* A69S, *ARMS2* del443ins54, and *CFH* Y402H were more likely to have nAMD (OR 3.13; 95% Confidence Interval [CI] 2.08–4.71 for *ARMS2* A69S, OR 3.28; 95% CI 2.17–4.95 for *ARMS2* del443ins54, and OR 2.08; 95% CI 1.08–3.99 for *CFH* Y402H). Genotype analysis showed significant differences between the nAMD and control groups for all polymorphisms (Table 2). The associations of *ARMS2* A69S and *ARMS2* del443ins54 (P<0.001) with nAMD were stronger than that of *CFH* Y402H (P=0.043).

In Table 3, it is shown that homozygous risk allele carriers at the *ARMS2* A69S polymorphism (OR 5.97; 95% CI 2.75–13.0) and *ARMS2* del443ins54 (OR 7.99; 95% CI 3.45–18.6) were both strongly associated with nAMD. For *CFH* Y402H, individuals with one copy of the risk allele were more likely to have nAMD than control (OR 2.47; 95% CI 1.19–5.11). These associations remained significant even after controlling for age, gender, smoking, body mass index and blood pressure.

Table 1 Baseline Characteristics of Participants

	nAMD (n=104)	Control (n=100)	P
Age, year			
Range (median)	45–83 (67)	49–99 (68)	0.16
Mean ± SD	66.3 ± 8.8	67.9 ± 7.7	
Sex			
Male	46 (44.2%)	45 (45.0%)	0.91
Female	58 (55.8%)	55 (55.0%)	
BMI (kg/m^2)			
Range (median)	15.2–37.1 (23.3)	15.2–36.8 (21.4)	0.002
Mean ± SD	23.7 ± 3.9	22.0 ± 4.1	
BMI distribution, n			
<18.5 kg/m^2	41 (39.4%)	40 (40.0%)	<0.001
18.5–22.9 kg/m^2	6 (5.8%)	34 (34.0%)	
23–24.9 kg/m^2	23 (22.1%)	16 (16.0%)	
>25 kg/m^2	34 (32.7%)	10 (10.0%)	
Sunlight exposure			
Indoor workplace	73 (70.2%)	72 (72.0%)	0.78
Outdoor workplace	31 (29.8%)	28 (28.0%)	
Smoking			
Never	73 (70.2%)	77 (77.0%)	0.27
Ever	31 (29.8%)	23 (23.0%)	
Blood pressure			
Normal blood pressure	56 (53.8%)	82 (82.0%)	<0.001
High blood pressure	48 (46.2%)	18 (18.0%)	

Abbreviations: nAMD, neovascular age-related macular degeneration; SD, standard deviation; BMI, body mass index; kg/m^2, kilogram/meter^2.

Table 2 Case–Control Frequencies of Alleles and Genotypes of SNP on *ARMS2* A69S, *ARMS2* del443ins54 and *CFH* Y402H

SNP			Allele Distribution (%)		Allele Association (P)	Crude OR (95% CI)		Genotype Distribution (%)		Genotype Association (P)	Crude OR (95% CI)	P (HWE)
			Case	Control				Case	Control			
ARMS2 A69S	G		61 (29.3%)	113 (56.5%)	<0.001	1.00 (reference)	GG	16 (15.4%)	34 (34.0%)	<0.001	1.00 (reference)	0.398
	T		147 (70.7%)	87 (43.5%)		3.13 (2.08–4.71)	GT	29 (27.9%)	45 (45.0%)		1.37 (0.64–2.92)	
							TT	59 (56.7%)	21 (21.0%)		5.97 (2.75–12.96)	
ARMS2 del443ins54	wt		59 (28.4%)	113 (56.5%)	<0.001	1.00 (reference)	wt	12 (11.5%)	32 (32.0%)	<0.001	1.00 (reference)	0.975
	indel		149 (71.6%)	87 (43.5%)		3.28 (2.17–4.95)	wt/indel	35 (33.7%)	49 (49.0%)		1.90 (0.86–4.21)	
							indel	57 (54.8%)	19 (19.0%)		7.99 (3.45–18.58)	
CFH Y402H	T		178 (85.6%)	185 (92.5%)	0.028	1.00 (reference)	TT	75 (72.1%)	86 (86.0%)	0.043	1.00 (reference)	0.528
	C		30 (14.4%)	15 (7.5%)		2.08 (1.08–3.99)	TC	28 (26.9%)	13 (13.0%)		2.47 (1.19–5.11)	
							CC	1 (1.0%)	1 (1.0%)		1.15 (0.07–18.65)	

Abbreviations: HWE, Hardy Weinberg equilibrium in control group; wt (wild-type), non-risk allele; indel, insertion/deletion.

In additional analyses, we documented significant interaction between *ARMS2* A69S and hypertension. Table 4 shows that individuals who had *ARMS2* A69S risk alleles and hypertension had significantly higher odds of nAMD than those with hypertension or *ARMS2* A69S risk alleles only (OR 9.53; 95% CI 3.61–25.1; P<0.001).

Discussion

In this study population, we documented that gene polymorphisms of *ARMS2* A69S and *ARMS2* del443ins54 were strongly and independently associated with nAMD. In contrast, we also documented that the association of CFH Y402H with nAMD was weaker than that of *ARMS2* A69S and *ARMS2* del443ins54. We also documented a synergistic effect between *ARMS2* A69S and hypertension meaning, that individuals with both *ARMS2* A69S risk alleles and hypertension had a significantly higher risk of nAMD. Findings from our study reconfirm that *ARMS2* genes are strongly associated with nAMD across different populations, at the same time suggest the existence of gene–hypertension interaction between this specific gene and hypertension.

We provided the first evidence of the associations of *ARMS2* A69S, *ARMS2* del443ins54, and *CFH* Y402H with nAMD in Indonesian population. There have been several studies from Asian population available for direct comparison.[15,30–32] *ARMS2* A69S gene polymorphisms have been consistently associated with nAMD in Malaysian,[33] Chinese Singaporean,[31] Thai,[30] Chinese,[34,35] Japanese,[36,37] Korean,[38] Indian,[32] and European populations.[39] It has also been reported that *ARMS2* A69S has stronger associations with nAMD than *CFH* Y402H,[40] which is comparable to our study findings. In addition to *ARMS2* A69S, results from our study showed that *ARMS2* del443ins54, also significantly associated with nAMD, which has been reported in Japanese, Caucasian, and Indian populations.[13–15,41] In contrast to *ARMS2*, associations between *CFH* Y402*H* gene variants and nAMD have been less consistent.[42] For example, *CFH* Y402H in Caucasian had a strong association with nAMD,[4,14,43,44] but studies from Asian showed a conflicting result. Xu et al,[34] Gotoh et al,[45] Okamoto et al,[46] Uka et al,[47] and Chen et al[48] showed a weak association of *CFH* Y402H with AMD while Lau et al[49] showed a contradictory result.

The role of *ARMS2* genes in nAMD has become a subject of interest for more than a decade.[10] *ARMS2* has been speculated to regulate the surface complement-mediated phagocytosis of cellular debris.[50] Micklisch et al[50] reported that

Table 3 Distribution of Unadjusted and Adjusted Odds Ratio for Risk Genotypes in *ARMS2* A69S, *ARMS2* Del443ins54 and *CFH* Y402H

Gene (SNP)	Genotype	OR (95% CI)	P	OR (95% CI)[a]	P	OR (95% CI)[b]	P
ARMS2 A69S	GG	1.00 (reference)	–	1.00 (reference)	–	1.00 (reference)	–
	GT	1.37 (0.64–2.92)	0.415	1.02 (0.46–2.29)	0.953	0.76 (0.30–1.94)	0.569
	TT	5.97 (2.75–12.9)	<0.001	5.89 (2.62–13.3)	<0.001	6.82 (2.52–18.5)	<0.001
ARMS2 del443ins54	wt	1.00 (reference)	–	1.00 (reference)	–	1.00 (reference)	–
	wt/indel	1.90 (0.86–4.21)	0.111	1.48 (0.65–3.38)	0.355	0.99 (0.38–2.61)	0.994
	indel	7.99 (3.45–18.6)	<0.001	7.39 (3.10–17.6)	<0.001	7.20 (2.56–20.2)	<0.001
CFH Y402H	TT	1.00 (reference)	–	1.00 (reference)	–	1.00 (reference)	–
	TC	2.47 (1.19–5.11)	0.015	2.73 (1.29–5.81)	0.009	3.84 (1.42–10.4)	0.008
	CC	1.15 (0.07–18.7)	0.923	0.61 (0.03–12.4)	0.751	0.94 (0.004–186)	0.982

Notes: [a]Adjusted for age and gender; [b]additionally adjusted for smoking, body mass index, and blood pressure.
Abbreviations: wt (wild-type), non-risk allele; indel, insertion/deletion.

Table 4 Interaction Analysis of *ARMS2* A69S and Hypertension

Category	OR (95% CI)	P-value	OR (95% CI)[a]	P-value
No hypertension & no risk allele	1.00		1.00	
Hypertension only	3.90 (2.06–7.40)	<0.001	4.51 (2.31–8.77)	<0.001
ARMS2 A69S only	2.83 (1.44–5.56)	0.002	2.81 (1.43–5.56)	0.003
Hypertension and *ARMS2*	9.53 (3.61–25.1)	<0.001	10.8 (4.00–28.7)	<0.001

Note: [a]Adjusted for age and gender.

decreases of the ARMS2 expression in AMD were associated with polymorphism of *ARMS2* A69S and del443ins54. Decreases in ARMS2 protein would result in drusen accumulation due to impaired cellular debris clearance.[50] Furthermore, a study by Yang and associates[51] suggested that *ARMS2* A69S risk allele may decrease antioxidant enzyme activity in end-stage AMD-specific induced pluripotent stem cells(iPSCs)-derived RPE model. RPE cells are exposed to intense photo-oxidative energy and excess oxygen, promoting reactive oxygen species (ROS). Decreases in antioxidant enzyme capacity lead to ROS accumulation, increasing oxidative damage contributed to AMD.

Some studies have suggested that inflammation may partly explain the link between AMD and *ARMS2* polymorphisms.[25,52] In iPSCs-derived RPE from AMD donor, Saini et al[52] showed that *ARMS2* risk allele increased the complement proteins and pro-inflammatory factors compared to iPSCs-RPE derived from healthy control. In addition, there was a study reporting that *ARMS2* del443ins54 was correlated with an increase in the serum high sensitivity C-reactive protein (hs-CRP) levels of nAMD subjects in a Japanese study.[25] High serum CRP is associated with the late stage of AMD in a systematic literature review and meta-analysis.[53] Serum CRP represents systemic inflammatory activity and is a marker of chronic low-grade inflammation.[53]

The present study also documented gene–hypertension interactions of the *ARMS2* A69S and hypertension. Hyman et al[54] reported that nAMD and hypertensive disease may have a similar underlying systemic process, as nAMD is linked to high diastolic blood pressure (OR: 4.4; 95% CI: 1.4–14.2). The involvement of oxidative stress accumulation processes in both nAMD and hypertension might explain these associations.

The strengths of our study included age-matched cases and controls, detailed clinical and eye examinations by retinal specialist using advanced multimodal imaging to confirm the diagnosis of nAMD and the application of PCR that ensured the accuracy of genetic assessment. However, several limitations were also noted. First, we

did not use indocyanine green angiography (ICGA) as the gold standard for nAMD diagnosis. Nevertheless, spectral-domain OCT had high sensitivity and specificity in distinguishing nAMD from polypoidal choroidal vasculopathy (PCV).[55–57] Diagnosis of nAMD based on fundus photography and spectral-domain OCT had more than 90% agreement when compared to ICGA,[58–60] thus reassuring the minimal bias in this study. Second, the hospital-based design of our study may have only captured the advanced profile of AMD patients, therefore limiting the representation of AMD in general population. Whether or not individuals with AMD from the general population have similar genetic associations remained questionable. Future population-based studies are warranted to address these questions.

In conclusion, our study highlighted a strong association of *ARMS2* A69S and del443ins54 in people with nAMD in Yogyakarta, Indonesia. This is the first study on nAMD's genetic risk factors and the first AMD research in Indonesia. Limited studies have been performed in Southeast Asia. Although our study found a weak relationship between the *CFH* Y402H polymorphism and nAMD risk, further studies are warranted to confirm the relationship of *CFH* Y402H and nAMD in Indonesian populations. Future work should have larger and more diverse sample sizes to allow subanalysis based on ethnic origin in Indonesia. Genetic information is important in the area of personalized medicine, and it may be useful as a baseline data to establish cohort studies of AMD clinical risk prediction scoring relevant to the Indonesian population.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

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The authors report no conflicts of interest for this work.

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