ORIGINAL ARTICLE

Strong Linkage Disequilibrium and Haplotype Association of Neovascular Age-related Macular Degeneration in Indonesian Patients

Supanji Supanji[1,2,3], Ayudha Bahana Ilham Perdamaian[1,4], Izzati Amayazifun Khalifa Farzana[1], Muhammad Bayu Sasongko[1,3], Angela Nurini Agni[1,3], Firman Setya Wardhana[1,3], Tri Wahyu Widayanti[1,3], Mohammad Eko Prayogo[1,3]

[1] Department of Ophthalmology, Faculty of Medicine Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia/ Dr. Sardjito General Hospital, Yogyakarta, Indonesia
[2] Ophthalmology Clinic, Military Air Force Central Hospital Dr. Suhardi Hardjolukito, Yogyakarta, Indonesia
[3] Ophthalmology Clinic, Dr. YAP Eye Hospital, Yogyakarta, Indonesia
[4] Doctoral degree of Biomedical science, Faculty of Medicine Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia

ABSTRACT

Introduction: The aim of this study was to investigate the linkage disequilibrium (LD) and haplotype of three most associated SNP with nAMD of 80 patients in Indonesia. **Methods:** All patients underwent standard ophthalmic tests including fundoscopy and optical coherence tomography. Genomic DNA was extracted using commercially available DNA isolation kits. Genotyping of rs11200638, rs1061170 and del443ins54 used polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) methods. The acquired genotype data were analyzed using Haploview and R package software. **Results:** Linkage Disequilibrium analyses showed high LD value in the 10q26 region of 80 patients with AMD and 85 controls. The PCR-RFLP showed TTA was the most frequent haplotype while GTG was the most associated haplotype in the study sample. **Conclusion:** There was a high LD in the 10q26 region and strong association in GTG haplotype of Indonesian patients with AMD.
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Corresponding Author:
Supanji, PhD
Email: supanji@ugm.ac.id
Tel: +62 274 560300

INTRODUCTION

Age-related Macular Degeneration (AMD) is a degenerative retinal condition recognized by drusen deposition, swelling, and thick macula. The exact cause of AMD manifestations remains unknown but several factors have been identified, with age, smoking, and genetic polymorphisms considered strong risk factors (1). AMD has two subtypes in the late stage with distinct characteristics: dryAMD which is recognized by geographic atrophy in the macula and wet or neovascular AMD which is recognized by swelling and neovascularization (2,3). Although Polypoidal Choroidal Vasculopathy (PCV) has similar features to nAMD, a recent study determined it as a subtype of the pachychoroid spectrum disease (4).

Based on systematic reviews (5,6) and genome-wide association analyses (7–9), nAMD had three of the strongest associated genetic polymorphisms. These genetic mutations are located on chromosome one (1q31) Complement Factor H (CFH; rs1061170) and chromosome 10 (10q26) ARMS2/HTRA1(del443ins54; c.*372_815del443ins54) and HTRA1 (rs11200638). The 10q26 locus is the most associated chromosome region in nAMD onset in Caucasians (10–12). Furthermore, most research found that the 10q26 locus were almost predictive i.e. near perfect linkage disequilibrium (D'>0.98) (13).

The rs11200638 is located precisely at High temperature requirement factor A1 (*HTRA1*) promoter. The rs11200638 location is near to rs10490924 and del443ins54 (ARMS2/LOC387715) (13–16). Also, being segregated together during chromosome crossing-over made almost all nAMD patient have similar mutation status with each other. One protein-coding gene that is associated with the development of nAMD, the Pleckstrin Homology Domain Containing A1 (*PLEKHA1*) is located

near rs11200638.

Epidemiological studies on other ethnic populations elicit similar results (13,17–29), but there are no similar analyses reported on Indonesian populations. No linkage disequilibrium (LD) and haplotype analyses were previously done in Indonesian patients with nAMD. The current research aimed to investigate the LD and joint effect of strong genetic loci in Indonesian nAMD cases.

MATERIALS AND METHODS

Study design

The study conceptualization, methodology, and data collection were approved by the Ethics Committee (KE/FK/864/EC at August 5, 2016; KE/FK/1109/EC/2017 at October 12, 2017; KE/FK/1108/EC/2018 at October 18, 2018). The diagnosis of the patients with nAMD and inclusion criteria were in accordance with the previous report (30).

Subjects were recruited from three hospitals in Yogyakarta (Sardjito Hospital, YAP Eye Hospital, and Hardjolukito Hospital) from 2016-2020. Subjects were aged > 45 years old with or without AMD. AMD diagnosis was conducted by a retinal specialist in the designated hospitals. All subjects underwent slit-lamp examination, indirect ophthalmoscopy, fundus photography and Spectral-domain Optical coherence tomography (SD-OCT). Subjects who were not willing to join the study and those with other retinal or choroidal inflammatory diseases were excluded from our study.

All participants were well explained about the purpose of study and the workflow before signing the informed consent form, undergoing blood collection and comprehensive ophthalmic tests.

Additional medical information such as hypertension, smoking, bodyweight, and body height were taken from the medical records or by direct anamnesis. Hypertension is defined as Systolic Blood Pressure (SBP) ≥ 140 mmHg, Diastolic Blood Pressure (DBP) ≥ 90 mmHg, or self reported use of antihypertensive medication. Smoking is defined as the activity of cigarettes smoking during the past 30 days. Using a calibrated scale, bodyweight was measured in kilograms and body height was measured in centimeters.

This study used 80 eligible late phase nAMD cases and 85 age-matched patients without nAMD or other retinal disease as controls.

Genotyping

The genomic DNA extraction and CFH, *ARMS2* and *HTRA1* fragment gene amplification was discussed in the previous report (30).

Statistical analysis

Linkage disequilibrium (LD) was estimated from previous research acquired data (30,31). All data including indel were reformatted into a linkage format. Analysis was done using Haploview (Broad Institute of MIT and Harvard, USA). Produced diagrams indicated the degree of each marker to be inherited together. Diagrams consisted of many squares in which each square represents the relationship of two markers. The color of each square was derived from the software as follows: white indicates D'<1 and log of the likelihood odds ratio (LOD) < 2 of two markers, pink (|D'|<1, LOD≥2), blue (|D'| = 1, LOD<2) or red (|D'| = 1, LOD≥2). The software also calculates the r^2 value, for which r^2 >0.5 indicates stronger association between alleles, and lower values of r^2 indicate weaker association between alleles. The threshold was based on r^2 (e.g., r2 ≥ 0.8)

The haplo.stats program was used to investigate each haplotype frequency and association. Haplo.stats is a protocol in the programming language R which uses an Expectation-Maximization algorithm to calculate the haplotype frequency and association with the disease onset (29, 30).

RESULTS

In the recent study, two markers spanning from position 10: 122454932 to 10: 122454935 showed high LD in one block consisting of rs10490924 (A69S; c.205G>T,) and *ARMS2* insertion-deletion (c.*372_815del443ins54) according to the LD diagram (Fig. 1.). The block has D'= 0.844 with LOD=52.21 and r2: 0.704.

Red or pink squares indicate the degree of LD between two markers whereas white squares indicate no

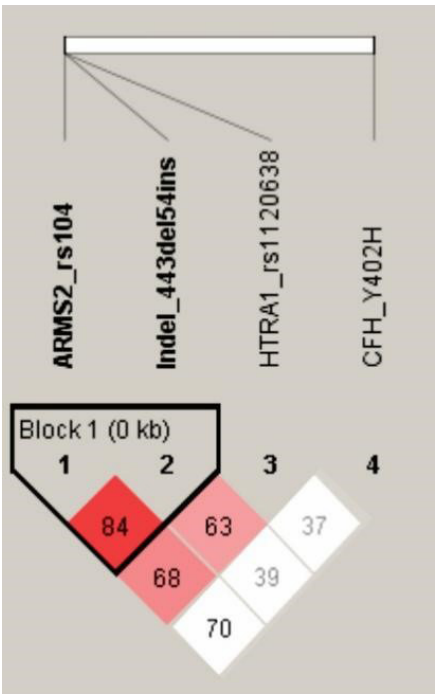

Figure I: The LD diagram of four strong associated loci to nAMD in Indonesia

significant LD. The LD value of *ARMS2* indel with *ARMS2* rs10490924 was D'= 0.68, whereas with rs1120638, LD was D'= 0.63.

After data adjustment for haplotype analysis, *ARMS2* rs10490924 was excluded since it did not follow the Hardy-Weinberg Equilibrium (HWE) equation. Comparisons of haplotype frequencies (Table I) and association (Table II) were done using only three single nucleotide polymorphisms (SNPs). From eight possible haplotype combinations, only five exceeded the minimum requirement for association testing.

Results without using any genetic factors or using haplotypes assuming dominant genetic traits were derived from haplo.stats. protocols in R. Haplotypes are analyzed separately and data were adjusted for age, gender, and smoking status. Only one haplotype (GTG) shows significant differences between cases and controls. The p-value of both assuming additive and dominant genetic traits were the same.

DISCUSSION

To our knowledge, this is the first study to investigate the

Table I: The haplotype frequency of three evaluated SNP

Haplotype	Haplotype Frequency (case)		Haplotype Frequency (control)		*p*-value	
	additive	dominant	additive	dominant	add	dominant
TTA	4.09×10^{-2}	4.1×10^{-2}	8.16×10^{-2}	8.16×10^{-2}	1.04×10^{-1}	1×10^{-1}
GTA	7.94×10^{-2}	7.94×10^{-2}	4.08×10^{-1}	4.08×10^{-1}	6.55×10^{-12}	2.46×10^{-14}
GTG	$1.77 \times^{10-1}$	1.77×10^{-1}	1.23×10^{-1}	1.23×10^{-1}	4.53×10^{-1}	3.82×10^{-1}
TCA	-	-	1.03×10^{-5}	1.03×10^{-5}	-	-
TTG	5.58×10^{-1}	5.59×10^{-1}	3.12×10^{-1}	3.13×10^{-1}	9.7×10^{-7}	9.43×10^{-6}
rare	1.17×10^{-1}	1.17×10^{-1}	4.08×10^{-2}	4.06×10^{-2}	4.70×10^{-3}	1.66×10^{-3}

Table II: Haplotype association with nAMD

Model Without Genetic Factors				
Covariate		OR (95% CI)	*p* value	
Constant		1.11	0.893	
Age group		7.69(0.86-68.52)	0.680	
Gender		0.72(0.35-1.49)	0.374	
Smoking		2.34(0.98-5.56)	0.054	
Worktime		1.11(0.33-3.69)	0.867	
Workplace		0.778(0.38-1.61)	0.500	
Blood Pressure		1.51(0.34-19.21)	≤0.001**	
BMI group		0.83(0.45-1.56)	0.559	
Models with Genetic Factors				
		Additive		Dominant
Covariate		OR (95% CI)	*p* value	OR (95% CI)	*p* value
Constant		314.19	Nan	141.18	Nan
Age group		10.11(0.95-107.14)	0.055	15.95(1.31-194.19)	**0.030***
Gender		0.81(0.35-1.88)	0.623	0.85(0.35-2.02)	0.690
Smoking		2.13(0.80-5.69)	0.133	2.08(0.75-5.79)	0.159
Worktime		1.47(0.35-6.16)	0.596	1.44(0.34-6.22)	0.622
Workplace		0.8120.36-1.87)	0.642	0.92(0.40-2.16)	0.853
Blood Pressures		4.48(2.03-9.86)	≤0.001**	4.67(2.06-10.61)	≤0.001**
BMI group		1.06(0.49-2.27)	0.886	1.17(0.53-2.56)	0.694
Gene (Chrom)	Haplotype	OR (95% CI)	*p* value	OR (95% CI)	*p* value
	TTA	Reference haplotype			
	GTA	0.72(0.35-1.47)	0.366	0.75(0.28-2.01)	0.568
	GTG	0.13(0.06-0.27)	≤0.001**	0.07(0.03-0.17)	≤0.001**
	TCA	1.23(0.46-3.30)	0.685	1.60(0.51-5.05)	0.423
	TTG	0.36(0.11-1.19)	0.093	0.41(0.11-1.46)	0.167
	rare	0.85(0.15-4.80)	0.854	1.21(0.18-8.05)	0.842

Abbreviations: BMI: Body mass index

LD and haplotype status towards nAMD in Indonesian patients. A total of 4 genetic variants were involved of which 3 were located within the tail domain of *ARMS2-HTRA1* located in the 10q24 region. Another gene identified was CFH Y402H, located on chromosome 1. These polymorphisms were associated with the onset of nAMD in Indonesian patients.

The research shows high LD value (D'= 84) implying the high heritability of two markers. Despite showing results not as high as other studies (Table III), the analysis yielded adequate evidence of unsolved genetic risk factors in the Indonesian population. Recombination rate in this block (r2: 0.7), which was also high, indicated these polymorphisms occurred frequently. Further analysis is needed to elucidate the causative risk factor. Since using a relatively small size population sample, especially the control, SNP combinations yield high r^2 values.

Other studies on many ethnic groups show high LD values for which many of them are nearly predictive. Most LD occur between *HTRA1* rs1120638 and *ARMS2* rs10490924 since they are not included in the *ARMS2* indel.

Haplotype frequency of wildtype was highest and the GTG was most associated with the onset of nAMD. These results may point to the effect of gene dysfunction on protein structure, but further study is needed to confirm these findings and identify the underlying pathogenic mechanism.

Haplotype frequency of wildtype (TTA) was highest (0.43), followed by GTA (0.14), GTG (0.24), TCA (0.08) and TTG (0.06). The GTG was most associated haplotype with the onset of nAMD ($p \leq 0.01$). The strong association of haplotype GTG without *ARMS2* rs10490924 might elucidate the indel was an independently causative risk factor for nAMD in Indonesian cases. Therefore, further analysis is needed to validate the current results.

CONCLUSION

The linkage disequilibrium value and joint effect of *ARMS2* rs10490924 and indel polymorphism had positive association with the incidence of neovascular age-related macular degeneration in Indonesian samples.

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Table III: The linkage disequilibrium analysis of 10q26 region in other study

No.	Authors	Ethnic	SNPs	LD score (D')
1	Jakobsdottir (12)	Caucasian	rs4146894 (PLEKHA1) and rs10490924 (LOC387715)	0.93
			rs4146894 and rs1045216	0.91
			rs1045216 and rs10490924	0.97
2	Rivera (32)	Caucasian (Germany)	rs10490924 and rs2421022	0.91
			rs10490924 and rs2901307	0.86
			rs10490924 and rs1045216	1
3	Sheila Fisher (32)	Caucasian (Russia)	rs2421016 (PLEKHA1) and rs10490924	controls: 0.68 AMD: 0.73
4	Losonczy (33)	Caucasian (Hungary)	rs10490924 and HTRA1 rs11200638	Controls: 3 out of 95 was equilibrium nAMD: 3 out of 105 was equilibrium
5	Leveziel (34)	Caucasian (France)	PLEKHA1 and LOC387715	Control: 0.328 nAMD: 0.636
			LOC387715 and HTRA1	nAMD: 1 controls: 0.98
6	Yule (35)	Asian (Chinese)	rs10490924 and HTRA1 rs11200638	Control: 0.98 nAMD: 0.87
7	Kaur (24)	Asian (India)	rs10490924 and rs11200638	0.90
			rs10490924 and rs2672598	0.80
8	DeAngelis (28)	Caucasian (USA)	rs10490924 and rs11200638	0.94
9	Hadley (36)	Caucasian (USA)	rs10490924 and rs11200638	0.98
			Del443ins54 and rs11200638	0.98
10	Liao (37)	Asian (Chinese)	rs10490924 and rs11200638	0.99
11	Schmidt (38)	Caucasian (USA)	rs10490924 and rs1882907	0.93
12	Francis (39)	Caucasian	rs10490924 and rs11200638	0.8

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