ORIGINAL ARTICLE

Smoking as a Risk Factor for rs10490924 Variant Age-related Macular Degeneration in Yogyakarta, Indonesia

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ABSTRACT

Introduction: The aim of this study was to investigate whether smoking status is a risk factor for age-related macular degeneration (AMD) with rs10490924 variant in Yogyakarta, Indonesia. **Methods**: A case–control study was conducted using 119 patients with AMD and 100 non-AMD. Whole DNA of all patients was extracted using a commercially available whole blood DNA isolation kit. Polymorphism in the *ARMS2/HTRA1* gene was genotyped using a restriction enzyme for rs10490924 (G>T). Details regarding the smoking status of the AMD and the non-AMD population were collected using a questionnaire. Logistic regression analysis was used to confirm the correlation between *ARMS2/HTRA1* allele and smoking status. **Results:** Genotyping results showed that the mean frequency of rs10490924 variant was higher in patients with AMD than in non-AMD patients. There was no statistical difference in smoking status between the AMD and the non-AMD patients. However, smoking increase patients risk of having AMD after adjusted to rs10490924 but cannot stated as cofounder factor. Patients with risk allele have six times possibility of having AMD than non-smoking controls. **Conclusion:** Smoking status increases the risk for AMD in rs10490924 variant of the *ARMS2/HTRA1* gene.

Keywords: Age-related macular degeneration, Smoking, Risk factors, High-temperature requirement A serine peptidase 1

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INTRODUCTION

Age-related macular degeneration (AMD) is manifested by vision impairment on central sight view, leading to complete blindness due to the macular damage. Till date, AMD remains one of the major causes of blindness among elderly people in developed countries (1). Wet AMD that comprises up to 10 % of all AMD cases is caused due to neovascularization, swelling, and retinal degeneration. These events are caused due to an abnormal accumulation of drusen. There are approximately 170 million people suffering from visual impairment or blindness caused by AMD in the world, among whom 11 million are in the United States (2). The number of people suffering from AMD has been increasing along with the growth of the elderly population, especially among people aged >50 years and Caucasian subjects.

Meanwhile, in Asia, which is inhabited by Mongoloid and Papuan minorities, the prevalence of this disease shows some differences. In the Japanese population, the prevalence of early AMD was found to be 12.7 %, whereas that of late AMD was 0.87 %, with the age of onset being ≥50 years (3). Results of the Singapore Malay Eye Study reported prevalence rates of 3.5 % and 0.30 % for early and late AMD at the age of 40–80 years, respectively (4). However, the exact data on the incidence of this disease are lacking in Yogyakarta, Indonesia. The prevalence of AMD has so far continued to increase due to the difficulties in early detection, prevention, and management.

Genetic factors have a significant contribution to the onset of AMD, but external factors also have an influence on its progression. Among the several genetic factors, polymorphisms in the high-temperature requirement A serine peptidase 1 (HTRA1), complement factor H (CFH), and age-related maculopathy susceptibility 2 (ARMS2) genes have been reported to be strongly associated with AMD. Along with genetic factors, smoking habit and consumption of a high-fat diet have also been listed. According to several epidemiological studies, smoking is one of the strong risk factors for AMD. Smoking also increases the possibility of developing neovascular form of AMD from atrophic AMD (5). This study was conducted to investigate the effect of *ARMS2/HTRA1*

(rs10490924) in association with the smoking behavior of patients with AMD in Yogyakarta, Indonesia.

MATERIALS AND METHODS

Study Participants and Procedures

Ethical approval was obtained from the Medical and Health Research Ethics Committee, Faculty of Medicine, Public Health and Nursing (FK-KMK) UGM, Universitas Gadjah Mada (EC.KE/FK/0536/EC/2019). A total of 119 patients with AMD and 100 non-AMD patients were recruited during the screening process. The screening was performed from 2016 to 2018. Informed consent was obtained from all participants before conducting eye examinations and blood collections.

We strictly recruited patients without any other retinal or systemic diseases to avoid interferences. Participants underwent standard eye examinations, including visual acuity assessment, fundus photography, and optical coherence tomography to confirm the diagnosis. A structured questionnaire was used to collect baseline data about the patients' lifestyle.

Collection of baseline data about the patients' lifestyle utilized a structured questionnaire which was verified by the Ethical Committee. Smoking habit was classified as either YES (current or occasional active smoking or those who had stopped smoking for <1 year), and NO (never or those who had stopped smoking >1 year). This case–control study was conducted at three DNA extraction and genotyping were performed at the Integrated Research laboratory, Faculty of Medicine, Public Health, and Nurse, Universitas Gadjah Mada.

Genotyping

Genomic DNA was extracted from peripheral blood samples using a commercially available DNA isolation kit (Cat. # GB100; Geneaid, USA). The concentration of the extracted DNA was measured using Nanodrop (Thermo Scientific, MA, USA). Single-nucleotide polymorphisms (SNPs) in *ARMS2/HTRA1* (rs10490924) were determined to evaluate the effect of polymorphism in patients with AMD.

The specific region of the gene was amplified using a conventional thermal cycler (Bio-Rad, California, USA) before performing electrophoresis on a 1% agarose gel. The run conditions and the reaction cocktail were implemented according to the instructions of the ready-to-use PCR kit (Kapa Biosystems, MA, USA) manufacturer. The procedures were as follows: initial denaturation at 95 °C for 3 min before 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and then extension at 75 °C for 30 s, followed by a final extension step at 75 °C for 10 min. The mixture contained 10 ng/µL DNA, 10 ng/µl forward and reverse primers, and 0.5 U/µL Taq polymerase in 25 µL of final concentration.

To amplify the specific region of the genes of interest, *ARMS2/HTRA1*, forward 5′-TACCCAGGACCGATGGTAAC-3′ and reverse 5′-GAGGAAGGCTGAATTGCCTA-3′ primers (Tm = 56.3° C) were used as described previously (6)–(8). The SNP in the *ARMS2/HTRA1* (rs10490924) amplicon was demonstrated by 4% agarose gel electrophoresis after 2 h using PVuII (New England Biolabs, MA, USA) restriction enzyme digestion at 37.5° C.

Sanger DNA Sequencing

To ensure that the SNPs obtained in this study were identical to the reference, a PCR product of each genotype was verified by Sanger sequencing. After acquiring the clear band on the agarose gel, the PCR product of each genotype (wild-type; heterozygote, and mutant) of *ARMS2/HTRA1* (rs10490924) was transferred to clean tubes and sealed. Forward and reverse sequencing of 50 µl PCR products were performed at a commercial DNA sequencing company (1st Base, Singapore). All genotypes of both AMD and non-AMD patients were successfully determined by restriction enzyme digestion for rs10490924: G>T in *ARMS2/HTRA1*. The obtained sequence data were consistent with the restriction enzyme digestion results in all the examined subjects.

Statistical Analysis

Statistical analysis used IBM SPSS Version 25 (IBM: New York, The USA) with Chi-square and Cochrane-Mantel-Haenszel test. All the processed data were described as mean (±) standard deviation and percentage (%). In this study, p values <0.05 was considered to be statistically significant.

RESULTS

The baseline data are presented in Table I. The number of female participants was higher than male participants in both the AMD and non-AMD groups. Among patients with AMD, there were 52 (55.30 %) females, and the non-AMD group consisted of 53 (54.10 %) females. These differences were not statistically significant (p = 0.863).

In terms of age, the maximum number of subjects in AMD group (41, 43.60 %) was found in the age group of ≥70 years, followed by the 60 to 69 year age group with 35 participants (37.20 %) and the 50 to 59 year age group with 18 subjects (19.10 %). While in non-AMD group, the highest number of subject was in the group 60 until 69 years with 50 subjects (51.00 %). The age difference between AMD and non-AMD was significant (p < 0.05). However, the OR showed a value of less than one in each subgroup, which indicated a protective factor against AMD instead of risk factor towards AMD. This might indicate abnormal sample distribution in each age subgroup.

Smoking was found to be insignificant between AMD

Table I: Baseline characteristics of AMD and non-AMD patients

Parameters	nAMD (n=94)	Non-AMD (n=98)	p	Odds Ratio
Gender				
Male	44.70%	45.90%	0.863	0.95 (95% CI: 0.54-1.68)
Female	55.30%	54.10%		
Age (years)				
≥70	43.60%	42.90%	0.026	0.36 (95% CI: 0.12 – 0.90)
60–69	37.20%	51.00%	0.0034	0.23 (95% CI: 0.08 – 0.68)
50–59	19.10%	6.10%	Ref	Ref
Smoking status				
Non-smoking	70.20%	76.50%	0.332	1.38 (95% CI: 0.73–2.63)
Smoking	29.80%	23.50%		
rs10490924				
Wild-type (GG)	17.00%	34.70%	Ref	Ref
Heterozygote (GT)	26.60%	43.90%	0.59	1.23 (95% CI: 0.57 – 2.67)
Risk allele (TT)	56.40%	21.40%	0.000013	5.36 (95% CI: 2.46 – 11.70)

and non-AMD group (p = 332). In both groups, the majority of participants did not smoke and their relatives were also nonsmokers. In the AMD group, 66 people (70.20%) were nonsmokers, and among the non-AMD patients, there were 75 nonsmokers (76.50%).

In AMD group, there were 56.40% of individuals with the risk allele (TT), 26.60% of them were carriers (GT), and 17.00% of subjects had the wild-type (GG) variant. Whereas, the corresponding proportions among the non-AMD patients were 21%, 45%, and 34%. Chi-square analysis revealed a significant correlation in the TT allele between AMD and non-AMD patients (p = 0.000013), with an OR of 5.36 (95% CI: 2.46–11.70). However, other alleles did not yield the same result. Further data analysis was done to interpret the statistical significance of smoking and *ARMS2/HTRA1* rs10490924 variant in AMD and non-AMD groups (Table II).

From Table II, patients with TT genotypes and smoking have 1.15 times higher possibility than non smoking TT patients. Patients with GT genotypes and smoking have 1.48 times higher possibility than non smoking GT patients. while patients with GG genotypes have 1.383 times higher than GG and no smoking. At general, same procedure in table I, patients AMD smoking have 1.383 times higher than non smoking AMD. Smoking become more tangible risk factor for AMD while adjusted to *ARMS2/HTRA1* genotypes.

Table II:Risk Estimate among AMD patients with rs10490924 variant of *ARMS2 HTRA1* adjusted to smoking status

Genotypes	Adjusted OR	95% Confidence Interval	
		Lower	Upper
TT_risk	1.149	0.355	3.721
GT_het	1.856	0.630	5.469
GG_wildtype	1.477	0.394	5.536
Crude OR	1.383	0.727	2.632

Adjusted OR for smoking were increase from 1.38 to 1.48 but not significant (P=0.26) (Table III). Crude OR different was 0.098 (1.48-1.38 = 0.098) or 7% which was less than 10%. These results indicate smoking not stated as co-founding factor for rs10490924 *ARMS2/HTRA1* according to Greenland (9). Cochrane-Mantel-Haenszel test was done to evaluate the association between smoking and SNP in both study groups by adjusting the analysis to each genotype of the *ARMS2/HTRA1* rs10490924 variant. While stratified to *ARMS2/HTRA1*genotype and smoking status, the OR was increase compared to non-adjusted data (Table IV). Smoker with high risk genotype had nearly six times higher possibility compared to nonsmoker with wildtype *ARMS2/HTRA1*. Higher than nonsmoker with high risk genotypes (OR: 5.76). While smoking status in carrier patients were not statically different.

Table III: Adjusted analysis of smoking status to AMD with *HtrA1* genotypes

Smoking status	OR (CI) unadjusted	P	OR (CI) adjusted	p
Smoking	1.38 (95% CI: 0.73-2.63)	0.24	1.48 (95% CI: 0.75-2.94)	0.26

Table IV: Stratification of AMD patients to *HtrA1* genotypes and smoking status

Smoking status	Genotype	OR (CI)	p
Smoker	Risk	6.62 (95% CI: 1.91-22.89)**	0.002*
Smoker	HET	2.12 (95% CI: 0.68-6.68)**	0.2
Nonsmoker	Risk	5.76 (95% CI: 2.31-14.37)**	0.0001*
Nonsmoker	HET	1.14 (95% CI: 0.45-2.89)**	0.77

* significant at p<0.05
** compared to non smoker wild type (GG) patient

DISCUSSION

To our knowledge, this is the first study discussing the association of genetic and wet AMD risk factors in an Indonesian population. Results of the study suggested that *ARMS2/HTRA1* polymorphisms were associated with AMD and smoking was a compounding factor for *ARMS2/HTRA1* in patients with AMD. The rs10490924 SNP occurs in the coding region, which produces an alanine-to-serine substitution mutation in codon 69 (A69S) in the putative *ARMS2* gene (10). Interestingly, the A69S variant does not compromise the function of *ARMS2* (11). In addition, *ARMS2* protein deficiency alone does not induce this disease. Although these mutant alleles compromise the function of *ARMS2*, they do not alter the expression of *HTRA1* (11). This

polymorphism might influence the biological pathways involved in AMD pathogenesis.

A normal *HTRA1* gene sequence encodes a serine protease, which plays a major role in assisting the degradation of cellular proteins. Human HTRA1 protein is an internal control that is secreted by various cell types, e.g. retinal pigment epithelium (RPE). The HTRA1 protein also inhibits the signaling of TGF-β, which is an important growth factor regulating cell differentiation and development of several tissues. In humans, there are four HTRA protein families, *HTRA1, HTRA2*, *HTRA3*, and *HTRA4*, which share similar structure and function. Polymorphisms do not disrupt the protein function but compromise the protective property of HTRAs later in the more advanced age (12).

Tobacco-based cigarette smoking is considered as a strong risk factor for AMD. Some reports demonstrate significant associations between current smoking status and age-related maculopathy. Cigarette smoke contains a vast diversity of toxic compounds that could trigger oxidative damage, vascular changes, and inflammatory processes. The most attentively observed cigarette smoke toxin, i.e. nicotine, has been shown to trigger angiogenesis and catecholamine release in experimental models. This process elevates platelet aggregation, which ultimately forms a thrombus. Nicotine is also known to trigger the synthesis of platelet-derived growth factor (PDGF). This PDGF protein could induce vascular smooth cell proliferation, which is associated with AMD. Inhalation of cigarette smoke might underline the RPE cellular changes in patients with AMD (13).

Comparative studies reported from Singapore and India also did not confirm a relationship between cigarette smoking and early AMD. Although smoking has been consistently and suitably associated with late AMD based on previous studies, the association between smoking and early AMD was not conclusive (14). Among the earlier studies, the Blue Mountains Eye study reported that the mean age of onset for incident early AMD was 64 years for current smokers ($p = 0.001$) and 70 years for past/nonsmokers ($p = 0.002$) (15).

In this study, smoking behavior alone was not associated with AMD. However, the sorting of the rs10490924 variant into three (wild-type, carrier, risk allele) groups led to higher odd of having AMD. A high percentage of smokers in the risk allele group could indicate a higher risk of neovascularization resulting in the incidence of wet AMD. However, it should be noted that *HtrA1* and smoking in the mainstream independently influenced neovascularization in a study on mice animal models (16). Therefore, this result is still inconclusive and requires further studies for confirmation.

Results of recent research show the associations of *ARMS2/HTRA1* with AMD were modified by smoking status. This result was with previous research which indicated that smoking activity increases the risk of developing AMD to four times compared to that in nonsmoker patients (13, 17). Smoking could initiate AMD through several possible pathways such as by triggering oxidative damage, angiogenesis, compromising blood circulation in the choroid of the eye, and activating the complement pathway and the immune system (19, 22). However, gen *ARMS2/HTRA1* polymorphism was not the only genetics factor for AMD. Another study on the CFH gene, involving people with the Y402H allele, reported that smoking habit is strongly related to a higher risk of developing AMD (23).

CONCLUSION

Based on our research, rs10490924 polymorphisms was strongly associated with AMD and smoking was a compounding factor for *ARMS2/HTRA1* in patients with AMD. Smoking status increases the risk for AMD in the rs10490924 variant of the *ARMS2/HTRA1* gene.

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