HtrA1 serine protease expression levels on age-related macular degeneration (AMD) patients in Yogyakarta

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Abstract. This research aims to investigate the HtrA1 serine protease circulating level of Age-related Macular Degeneration (AMD) patients in Yogyakarta, Indonesia. This study was conducted from January to August 2019 which included 38 AMD patients and 16 Non-AMD patients/controls (two groups). Baseline data and blood sample were collected. ELISA assay was used to measure the HtrA1 serine protease circulating level on both groups. SNP genotyping of rs10490924 was using restriction enzyme digestion. This study used The IBM SPSS® version 24 (Chicago, The USA) to determine the relationship between HtrA1 expression level and AMD incidence. AMD patients had higher HtrA1 serine protease level (35.31) than controls (30.08). However, there is no association found between HtrA1 serine protease level and AMD incidence (p-value>0.05, CI 95 %). However, HtrA1 serine protease did not associate positively to AMD incidence in Yogyakarta samples. Further analysis by grouping AMD patient based on the rs10490924 genotype show no statistical correlation between HTRA1 to the incidence of AMD. This result might be due to the lack of samples in the study groups. Future studies with larger number of samples are advised to better see the association between Htra1 serine protease level and AMD incidence.

1 Introduction

Age-related Macular Degeneration (AMD) is a degenerative disease that compromise the photoreceptor cells, especially at the macula, a specific area of the retina [1]. To date, AMD is an irreversible eye disease that causes central vision problems due to the macular damage in one or both eyes. AMD is one of the major causes of blindness among the elderly people. Wet AMD which comprise up to 10 % of all case, caused by neovascularization, swelling, and retina degeneration. These events in wet AMD caused by drusen abnormal accumulation. Approximately 170×10^6 people are suffering impairment acuity or blindness caused by AMD in the world, and among 11×10^6 of it is in the United States. The prevalence of this disease was increases along the growth of the elderly population, especially in people above 50 yr old Caucasian.

The loss of photoreceptor cells initiates the pathogenesis of early AMD is preceded by the development of deposits (called drusen) beneath the retina. Accumulation of drusen ultimately leads to irreversible central vision loss. The presence of drusen is considered diagnostic for early or dry AMD. It is called dry AMD due to non-exudative characteristic and atrophy caused by yellowish debris of the waste products of the eye cells. The development of drusen is thought to be related to inflammatory process which backed up by the fact that the later stage exudative type involves inflammatory process. The presence of key proteins such as complements, amyloid-β, and IgG is evidence to show inflammatory response involvement [2].

Previously, researchers attempt to correlate a vast diverse factor (such as age, gender, race, genetic, etc.) to the insidency of AMD to illuminate the progression causes. Commonly, the prevalence of AMD increases as much as the growth of the elderly population [3]. While on the process before blindness occurs, symptoms of AMD are often not perceived. In Indonesian case, the AMD patients commonly come with advance diseased manifestation. Risk factors for the development of AMD are divided to non-modifiable risk factors i.e. genetics and modifiable risk factors i.e. lifestyle.

The pathogenesis of AMD was not entirely known, previous research findings and the scientist consensuses presume that AMD closely related to inflammation and Reactive Oxygen Species (ROS) stress. Retinal oxidative damage can occur due to the formation of reactive oxygen species (ROS) from oxidation in the mitochondria. Macula is very susceptible to oxidative damage because the photoreceptor inside has more mitochondria, while in the outside contains many fatty acids to leak ROS.

The *HTRA1* (HtrA serine peptidase 1) gene encodes serine proteases expressed in human RPE and has the

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main role in degrading the denatured protein. Selected SNP in this study (rs10490924) were located on 10q26. The ARMS2/HTRA1 (rs10490924) polymorphism occurs in the coding region for *ARMS2* and partially *HTRA1*.

This region encodes both partially exon one HtrA1 gene and the entire region of ARMS2 (LOC387715) gene [4]. Study show that HtrA1 expression is influence by ARMS2 InDel [5].

rs10490924 SNP produces an alanine-to-serine substitution in codon 69 (A69S) mutation in the putative ARMS2 gene [6]. Interestingly, A69S variant does not compromise ARMS2 function [7]. Furthermore, ARMS2 protein deficiency alone is not induce the disease, although those common risk alleles alter ARMS2 but not HTRA1 expression [4]. These polymorphisms might influence in different biological pathways of AMD pathogenesis.

Based on the epidemiological data, Genetic factors including gene polymorphisms of *age-related maculopathy susceptibility 2* (*ARMS2*), *high temperature requirement protease A1* (*HTRA1*), and *complement factor H* (*CFH*) strongly associated to the onset of AMD. However, there is no data regarding the contribution of those genetic factors to AMD patients, particularly in Indonesia.

This research aims to investigate the HTRA1 circulating level on Age-related Macular Degeneration (AMD) according to the rs10490924 genotype in Yogyakarta, Indonesia.

2 Methods

2.1 Study participants and procedures

Ethics approval was received from the Medical and Health Research Ethics Committee (MHREC), Faculty of Medicine, Public Health and Nursing (FK-KMK) UGM, Universitas Gadjah Mada. During eye screening, 38 AMD and 16 control patients were recruited. Screening was performed from January until August 2019. Informed consent was obtained from all participants before eye examination and blood collection. We strictly recruit patients without any other retinal or systemic disease to avoid interference. Participants undergo standard eye examination, including visual acuity assessment, fundus photographs, and optical coherence tomography (OCT) to diagnose AMD or healthy eye verification for control. A structured questionnaire was used to collect baseline data about lifestyle including smoking status (Active or passive) and indoor/outdoor working activity of the AMD and control patients. This cross-sectional case-control study took place in dr. Sardjito Central General Hospital, dr. S.Hardjolukito Air Force Main Hospital, and Dr. Yap Eye Hospital, Yogyakarta. Whereas DNA extraction and genotyping were performed in laboratory of Molecular Biology, FKKMK, UGM.

2.2 Genotyping

Genomic DNA was extracted from peripheral blood sample using commercial DNA isolation kits (Cat. # GB100; Geneaid, USA). Extracted DNA concentration was measured using nanodrop (Thermo Scientific, MA, USA). Single nucleotide polymorphism (SNP) on ARMS2/HTRA1(rs10490924) were used to show the polymorphism effect on AMD patients.

The specific region of interest was amplified using conventional thermal cycler (Bio-Rad, California, USA) prior to electrophoresis in 1 % agarose gel. Run condition and reaction cocktail were followed ready-to-use PCR kit (Kapa Biosystems, MA, US) manufacturer as follow: initial denaturation of 95 °C for 3min before 35 cycle of 95 °C denaturation for 30 s, 60 °C annealing for 30 s, and 75 °C extension for 30 s subsequent 75 °C final extension for 10 min. Mixture contains 10 ng/µl DNA, 10 ng/µL forward and reverse primer and 0.5 U/µl Taq-polimerase on 25 µL of final concentration. To amplify the specific region of genes of interest. HTRA1 (rs10490924) specific region was amplified using Foward: 5'-TACCCAGGACCGATGGTAAC-3' and Reverse: 5'-GAGGAAGGCTGAATTGCCTA-3' primer (Tm=56.3 °C).

The SNP on HTRA1 (rs10490924) amplicon showed 2 % agarose gel electrophoresis after overnight PVuII (New England Biolabs, MA, US) restriction enzyme digestion at 37.5 °C.

2.3 Sanger DNA sequencing

To ensure the SNP positions acquired in this research were identical with reference, PCR product from both AMD and patients were verified by sanger sequencing. After acquired clear band agarose electrophoresis, PCR products of each genotype (wild type; heterozygote, mutant) of the HtrA1/ARMS2 (rs10490924), were placed into clean tubes and sealed. Forward and reverse sequencing of 50 µL PCR products were quoted to commercial DNA sequencing company (1st Base; Singapore).

2.4 ELISA assay

Plasma was retrieved by collecting the upper layer of centrifuged whole blood at 1 000xg for 15 min. ELISA assay was done by followed the manufacturer protocol (Finetest®, Wuhan Fine Biotech, Wuhan, China). First, 100 µL of samples and standard were placed into 96x wells subsequent incubated 37 °C for 90 min. Wash two times then add 100 µL Biotin prior incubated at 37 °C for 60 min. Wash three times then at streptavidin conjugate buffer before being incubated at 37 °C for 30 min. Wash five times then add 90 µL TMB substrate buffer before being incubated at 37 °C for 10 min to 20 min (depend on the colour changing) in the dark. Immediately add 50 µL stop solution when the colour perfectly changed according to the standard. Placed into microplate reader immediately and read at 450 nm wavelength. Interpolated the O.D. into ng/ml using CurveExpert 1.4 software.

2.5 Statistical analysis

All acquired data was analysed using The Mann-Whitney Test (SPSS) for unevenly distributed data to see any association between serum serine protease level from HTRA1 gene expression and the incidence of AMD. Additional statistical analysis for checking the association between genotype and AMD incidence in both groups was done using Crosstabs (Chi-square tests).

3 Results

The initial data used for statistical analysis included 38 patients and 16 controls. However, after preliminary analysis using normalization test to check for data distribution (Shapiro-Wilk), the data was found to be abnormal (heterogenic [<0.05]). This could indicate the lack of samples in one of the arms compare to the other. Data normalization was done to balance both arms by reducing the number of samples from AMD group into only 16 (initially 38) samples using SPSS software (sample random selections). Therefore, both arms have the same number of samples, which is 16 samples in both arms.

After reducing the samples, the data was better distributed even though it was still abnormal in AMD patient group. Mann-Whitney Test for unevenly distributed data was done to see any association between serum serine protease level from HTRA1 gene expression and AMD incidence.

The mean value of serum protease level for AMD patients was 35.31 ± 15.73 SD), while the controls were 30.08 ± 12.78 SD. There is no positive association found between the serine protease level and AMD (*p-value> 0.05*, CI 95 %).

Additional statistical analysis for checking association between HTRA1 genotype (Figure 1) which confirmed by sanger sequencing (Figure 2) and AMD incidence in both groups was done by using Crosstabs (Chi-square tests; Table 1). But unfortunately, the results were consistently insignificant in all groups of alleles (*p-value <0.05*, CI 95 %).

Table 1. Crosstabs analysis of rs10490924 *ARMS2/HTRA1* genotypes

Genotype		Diagnosis		Total
		Control	AMD	
Risk	Count	3	7	10
	Expected	5	5	10
	%	30	70	100
Heterozygote	Count	9	7	16
	Expected	8	8	16
	%	56.3	43.8	100
Wildtype	Count	4	2	6
	Expected	3	3	6
	%	66.7	33.3	100
Total	Count	16	16	32
	Expected	16	16	32
	%	50	50	100
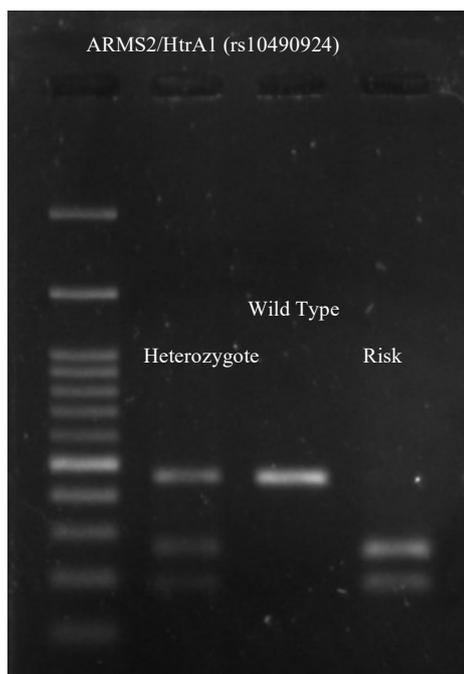
Fig. 1. Fluorescent-stained agarose gels of PCR and PCR-restriction fragment length polymorphism (RFLP) of HtrA1 (rs10490924) polymorphism. agarose gel electrophoresis after PVuII overnight incubation produce digestible fragment (G/G), undigestible fragment (T/T), and heterozygote (T/G)
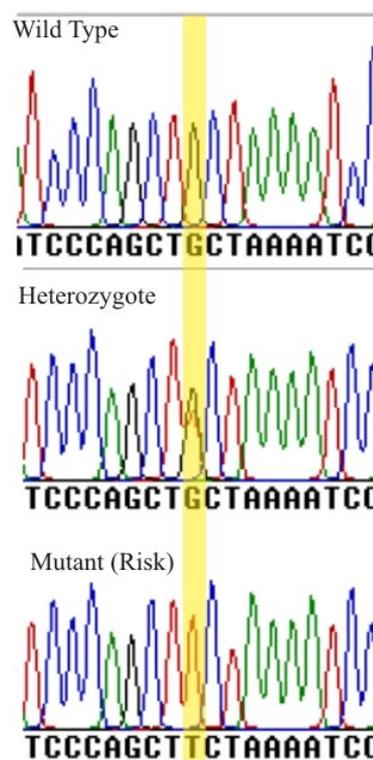
Fig. 2. HtrA1 polymorphism (rs10490924) showed by corresponding sequence chromatogram of sanger sequencing verification produce wild (G/G), mutant (T/T), and heterozygote (T/G) allele.

4 Discussions

To our knowledge this is the first report of association between HtrA1 circulating level and neovascular AMD in Indonesian population. This study results suggested that HtrA1 were associated with AMD, both of which demonstrated a higher susceptibility to AMD.

AMD patients had higher HtrA1 serine protease level (35.31 ± 15.73 SD) than controls (30.08 ± 12.78 SD). However, there is no association found between HtrA1 serine protease level and AMD incidence (*p-value>0.05,* CI 95 %). Dividing patient and control group into risk, carrier, and wildtype subgroup remain insignificantly difference.

Other studies show an association of circulating level of HtrA1 with several condition such as frailty of older adults [8]. HTRA1 also to be found increased during preeclampsia [9].

Invitro studies show a higher HTRA1 expression level on rs10490924 variant than wildtype. Its target protein cleave ext also followed this high concentration of HTRA1. EFEMP1, an extracellular matrix protein and thrombospondin 1 (TSP1), an inhibitor of angiogenesis [10].

Naturally, a normal sequence HTRA1 gene encodes a serine protease which is major role to help cell degradation. HtrA1 is one of importance internal control which produced by Retinal pigment epithelium (RPE) [10]. The HtrA1 protein also inhibits signalling of TGF-β [11] which is an important growth factor regulating cell differentiation and development of many tissues [12]. From that perspective, HTRA1 may be a proper candidate for AMD biomarker than ARMS2.

This result might be due to the lack of samples in the study groups. Future studies with larger number of samples are advised to comprehend the association between Htra1 serine protease level and AMD incidence.

5 Conclusion

Based on currents research, HtrA1 circulating level was not different between patient and control group. No statistical different was found in between wild type, heterozygous, and mutated genotypes of rs10490924.

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