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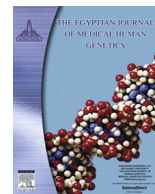


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Original article

R102G polymorphism of the *complement component 3* gene in Malaysian subjects with neovascular age-related macular degeneration

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ABSTRACT

Background: Genetic and environmental factors are known to be risk factors in development of neovascular age-related macular degeneration (nAMD). Genetic factors such as polymorphisms in the complement component pathway genes might play a role in pathogenesis of nAMD and has been studied in various populations excluding Malaysia.

Aim of the study: To determine the association of the R102G polymorphism of the *complement component* (C3) gene in nAMD subjects.

Patients and methods: A total of 301 Malaysian subjects (149 case and 152 controls) were recruited and genotyped for the R102G (rs2230199) variant of the C3 gene. Genotyping was conducted using the PCR-RFLP method and association analysis was conducted using appropriate statistical tests.

Results: From our findings, no significant association was observed in the allele distribution of C3 R102G between nAMD and controls (OR = 1.42, 95% CI = 0.77–2.62, P = 0.268). A further analysis that compared three genetic models (dominant, recessive and co-dominant) also recorded no significant difference (P > 0.05). These findings could be due to the low frequency of the GG variant in the case (4.7%) and control (1.3%) groups, compared to the normal variant CC, which is present in 91.3% of case and 92.8% of control alleles.

Conclusion: The present study showed no evidence of association between C3 R102G polymorphism and nAMD in Malaysian subjects.

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1. Introduction

Age-related macular degeneration (AMD) is a complex multifactorial and heterogeneous disease that results in progressive degeneration of the macula [1]. The most advanced form of AMD, is neovascular (wet or exudative) (nAMD), which can lead to a sudden deterioration of vision due to a hemorrhage or fluid leaking

from abnormal blood vessels developing underneath the macula [2]. Although the etiology of AMD remains elusive, due to the presence of inflammatory proteins and immune-mediated processes in drusen, a pathological deposit that forms between the Bruch's membrane and retinal pigment epithelium (RPE), inflammation has been suggested as the pathogenesis of AMD [3].

Increasing numbers of molecular studies have emerged providing better insights into the molecular pathology of AMD, such as single nucleotide polymorphisms (SNPs) of candidate genes related to inflammatory processes in the complement system involving the biogenesis of drusen [4]. The complement system is a complex of many enzymes and regulators; however, complement component activation, in particular C3a, the cleavage product of comple-

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ment component 3 (C3), has been found to be active in drusen and has been proposed to be a risk factor in AMD [5]. The progression of choroidal neovascularization (CNV), cell degeneration and the formation of drusens in AMD has been suggested due to dysfunctional in the complement system [6], and the plasma complement concentrations of arginine (Arg) that is terminally cleaved from the C3a parent molecule has also been reported to be higher among AMD subjects compared to normal subjects [7], reflecting C3 gene as a possible risk factor to AMD.

The human C3 gene is located on chromosome 19 and the R102G (rs2230199 C > G), a non-synonymous SNPs of the C3 gene which causes an amino acid change from arginine to glycine, has been studied in various populations but presents contradictory findings [8,9]. These inconsistent results encourage further investigation into the correlation between C3 and AMD among the Malaysian population, especially among the nAMD subtype. Moreover, it has been reported that genetic heterogeneity of complex diseases is common in different ethnic groups [10]; thus, the variants of an Asian population might not be similar to a population of European descent. Furthermore, to our knowledge, there are not yet any studies on C3 gene polymorphisms and nAMD in Malaysians, which led us to conduct this study on the effects of C3 R102G gene polymorphisms and their association with nAMD subjects in Malaysia.

2. Patients and methods

2.1. Patients selection

This study includes 149 nAMD patients and 152 controls recruited from the Ophthalmology Clinic in two tertiary centers, Hospital Selayang and Universiti Kebangsaan Malaysia Medical Center (UKMMC). Sample size was calculated by aiming at 80% statistical power. Prior to recruiting the subjects, ethical approval was obtained from the institutional review board of Universiti Putra Malaysia (UPM), Universiti Kebangsaan Malaysia (UKM) and Medical Research Committee (MREC) of the National Medical Research Registry (Ethics No: NMRR-14-1176-21475); and the work has been carried out in accordance with The Code of Ethics of The World Medical Association (Declaration of Helsinki) for experiments in humans. Consent was obtained from all subjects before recruiting them in the study and socio-demographic and medical history of each subject was recorded.

Subjects received a comprehensive ophthalmic examination by ophthalmologists in the respective centers and diagnosis of nAMD was confirmed based on fundus examination, optical coherence tomography (OCT), fluorescein angiography (FA) and clinical examinations.

2.2. Inclusion and exclusion criteria

Subjects aged > 50 years, diagnosed with nAMD after undergoing comprehensive ophthalmic examinations and presence of choroidal neovascularization (CNV) in either or both eyes, were included in this study. CNV resulting from the other causes and the other retinal diseases were excluded. Subjects aged > 50 years with no clinical evidence of nAMD or retinal disorders were recruited as control samples.

2.3. Gene and SNP selection

Twenty five associated candidate genes for AMD were identified from recent AMD genome wide association studies and previous association studies [11,12]. Using the search terms: age-related macular degeneration, AMD, SNP, association study or polymor-

phism; literature reviews were obtained from the online journal databases. R102G of the C3 gene was one among the significantly associated gene with AMD in many populations (Table 1) but not studied among Malaysians and thus, C3 gene polymorphisms was selected as a candidate SNP in the present study. Also, the SNP was selected based on the functional significance, location and minor allele frequency of >10% [13,14].

2.4. Genotyping

Polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) method was used to genotype the rs2230199 (R102G) SNP of C3 gene. Peripheral venous blood was collected from all subjects into 5 mL EDTA tubes. Genomic DNA was extracted from collected blood samples using QIAamp DNA blood mini kit (Qiagen, Valencia, CA, USA). The PCR was then carried out in a thermo cycler (Thermo Fisher Scientific, Waltham, MA USA) in a total volume of 25 µl with the following primers: forward: 5'-CTCACCTGTGGAGCCAGGGGTGT A-3' and reverse: 5'-CCAAAACGGCCACCTCGGAAGACC-3. Genomic DNA amplification was performed by performing 35 cycles of denaturing at 95 °C for 60 s, annealing at 55 °C for 60 s and extending at 72 °C for 30 s, followed by a single final extension at 72 °C for 5 min. The *HhaI* restriction enzyme (New England Biolabs, Beverly, MA, USA) was then used to digest the amplified product at 37 °C for 3 h. PCR and digested products were separated on a 3% agarose gel then visualized using a UV alpha imager (Alpha Innotech, San Leandro, CA).

2.5. Statistical analysis

Student's *t*-test and chi-square test were used to analyse the variables. Frequencies of alleles were estimated using the allele counting method and Hardy-Weinberg equilibrium assessed. The association of rs2230199 to nAMD was evaluated by logistic regression test using genotype and allele frequencies. A logistic regression model that assumed a co-dominant, dominant and recessive model was used to calculate the odds ratio (ORs) and 95% confidence intervals (CIs) of nAMD; the case and control groups were compared. All statistical analyses were performed using SPSS version 21.0 (SPSS Inc., Chicago, IL, USA) and $P < 0.05$ was considered to be statistically significant.

3. Results

The case group reported a significantly higher mean age (68.6 ± 8.47 years) compared to the control group (64.8 ± 10.21 years, $P < 0.001$). The ethnicity of the subjects also differed significantly ($P = 0.009$) with most nAMD cases being ethnically Chinese (57%) whereas Malay presented the highest ethnicity in the control group (42%). Among the co-morbidities recorded, diabetes was significant among controls (13%, $P = 0.036$) while hypertension was significant among nAMD cases (36%, $P = 0.001$). There is also a significant difference frequency of smoking, with cases reporting 37% and controls 19% ($P < 0.001$). Gender and diabetes with hypertension did not show any significant difference between cases and controls ($P > 0.05$). Table 2 represents the demographic features of the study population.

Genotyping using the PCR-RFLP method was performed as shown in Fig. 1. The high- risk GG genotype of rs2230199 was recorded to be low in both cases and controls, with frequency of 7 (4.7%) and 2 (1.3%), respectively. The allele distribution of the G allele was also very low in cases and controls, at 6.7% and 4.3%, respectively. Further analysis showed that when compared to low-risk CC genotype and C allele, neither the GG genotype or G

Table 1
Association of C3 R102G gene polymorphism in other studies.

Author	Location	Ethnicity	Sample (cases/controls)	Cases ^a		Controls ^a		Association ^b
				W	M	W	M	
Yates et al. [16]	England, Scotland	Caucasian	603/350	848 (0.72)	332 (0.28)	555 (0.80)	137 (0.20)	Yes
Spencer et al. [22]	USA	Caucasian	701/286	NA	203 (0.29)	NA	60 (0.21)	Yes
Scholl et al. [23]	USA	Caucasian	99/612	143 (0.73)	51 (0.26)	964 (0.83)	204 (0.17)	Yes
Cui et al. [18]	China	Asian	150/161	297 (0.99)	3 (0.01)	321 (0.99)	1 (0.03)	No
McKay et al. [24]	Northern Ireland	Caucasian	437/436	615 (0.70)	259 (0.30)	679 (0.78)	193 (0.22)	Yes
Zerbib et al. [25]	France	Caucasian	1080/406	1600 (0.74)	560 (0.26)	639 (0.79)	173 (0.21)	Yes
Chen et al. [26]	San Diego	Caucasian	1335/509	NA	0.28	NA	0.19	Yes
Kim et al. [21]	Korea	Asian	153/197	306 (1.00)	0 (0)	394 (1.00)	0 (0)	No
Tian et al. [17]	China	Asian	535/469	1060 (0.99)	4 (0.01)	928 (0.99)	2 (0.01)	No
Wu et al. [19]	China	Asian	165/216	328 (0.99)	2 (0.01)	428 (0.99)	4 (0.01)	No
Contreras et al. [27]	Mexico	Caucasian	273/201	NA	NA	NA	NA	Yes
Havvas et al. [28]	Greek	Caucasian	120/140	NA	40 (0.34)	NA	30 (0.22)	Yes
Present study	Malaysia	Asian	149/152	278 (0.93)	20 (0.07)	291 (0.96)	13 (0.04)	No

W = normal allele; M = mutant allele; NA = data not available.

^a Data are reported as number of subjects with percent in parentheses.

^b Association among AMD patients vs. controls.

Table 2
Demographics of study population.

		Cases (n = 149)	Controls (n = 152)	P ^a
Age (years)		68.6 ± 8.47	64.8 ± 10.21	<0.001 ^{b,*}
Gender	Male	95 (64.0)	112 (74.0)	0.063
	Female	54 (36.0)	40 (26.0)	
Race	Malay	51 (34.0)	64 (42.0)	0.009 ^c
	Chinese	85 (57.0)	62 (41.0)	
	Indian	13 (9.0)	26 (17.0)	
Co-morbidities	DM	9 (6.0)	20 (13.0)	0.036 ^c
	HPT	53 (36.0)	29 (19.0)	0.001 ^c
	DM + HPT	40 (27.0)	38 (25.0)	0.715
Smoking		56 (37.0)	28 (19.0)	<0.001 ^c

Data presented as mean ± SD for mean age and data are reported as number of subjects with percent in parentheses.

DM = diabetes mellitus; HPT = hypertension.

^a Chi-square test, P < 0.05.

^b Student *t*-test, P < 0.01.

^c Significant P-value, P < 0.05.

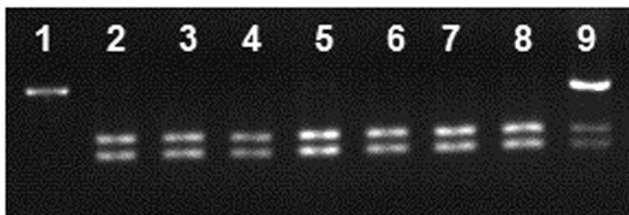


Fig. 1. PCR-RFLP product for C3 R102G gene polymorphism on 3% agarose gel. Lane 1 shows RFLP product for homozygous GG at 300-bp. Lane 2 until lane 8 shows RFLP product for homozygous CC at 133-bp and 167-bp. Lane 9 shows RFLP product for heterozygous CG at 133-bp, 167-bp and 300-bp.

allele were significantly associated with an increased risk of nAMD ($P = 0.073$, $P = 0.268$, respectively) (Table 3).

Including age, ethnicity, diabetes, hypertension and smoking as covariates in the logistic regression model did not substantially change the significance of the association (Table 4). None of the three genetic models showed significant differences between subjects with $P = 0.718$ in a dominant model, $P = 0.064$ in a recessive model and $P = 0.068$ in a co-dominant model. However, in a recessive genetic model, the OR 4.67 (95% CI = 0.91–23.87) for subjects carrying the GG mutant genotype was higher when compared to CG + CC genotypes; this was followed by a co-dominant model OR 2.14 (95% CI = 0.95–4.84) for subjects carrying the GG mutant genotype compared to those with the normal CC genotype. The

dominant model that compares between normal CC genotype with CG + GG genotypes had the least OR with 1.18 (95% CI = 0.48–2.91).

4. Discussion

This study evaluated the association of R102G SNPs (rs2230199) of C3 with nAMD in a Malaysian population and observed no significant association to nAMD even after adjusting for confounding factors, as the risk-associated G allele was recorded to be very low in cases and controls. Based on findings that suggest R102G is strongly correlated with AMD, the R102G polymorphism was included in the present study and compared to other polymorphisms in the C3 gene. Amino acid changes from R102G may alter configuration of the macroglobulin ring causing alteration in the binding to other complement proteins or pathogenic cell surface [8].

The R102G also produces two electrophoretic allotypes of C3, the C3F (fast variant) and C3S (slow variant) [15]; it is the C3F allotype that has been associated with AMD [16]. Association between R102G SNPs in C3 has also been extensively studied in other populations, but with contradictory findings, as summarized in Table 1.

The present finding is consistent with other relevant Asian studies such as Tian et al., Chinese population study that also recorded a low number of risk G allele and found no significant association to AMD [17]. Findings from a meta-analysis of R102G and AMD that included 15 independent case-control studies also concluded

Table 3
Genotypes and allele frequencies and odds ratios in cases and controls.

Genotype	Cases	Controls	OR (95% CI) ^a	P ^a
CC	136 (91.3)	141 (92.8)	1.00 (Reference)	
CG	6 (4.0)	9 (5.9)	0.55 (0.17–1.71)	0.299
GG	7 (4.7)	2 (1.3)	4.46 (0.87–22.87)	0.073
C allele	278 (93.3)	291 (95.7)	1.00 (Reference)	
G allele	20 (6.7)	13 (4.3)	1.42 (0.77–2.62)	0.268

CI = confidence interval; OR = odds ratio.

^a Logistic regression analysis.

Table 4
Genotype association of nAMD based on dominant, recessive and co-dominant models.

Genetic model	OR (95% CI) for nAMD ^a	P-value ^a
Dominant (CC vs. CG + GG)	1.18 (0.48–2.91)	0.718
Recessive (GG vs. CG + CC)	4.67 (0.91–23.87)	0.064
Co-dominant (GG vs. CC)	2.14 (0.95–4.84)	0.068

CI = confidence interval; OR = odds ratio.

^a Logistic regression analysis, adjusted for age, ethnicity, diabetes, hypertension and smoking.

that the rs2230199 C > G SNP was an increased risk among the Caucasians but not among Asians, as the risk G allele was recorded low in most Asian populations [6]. Other studies that reported no significant association of rs2230199 and AMD are from China [18–20] and Korea [21].

Although the risk variant of R102G is rare among Asians, the frequency is almost 20% among the European descents [9], thus suggesting that the variant may be associated with development of AMD. This is further supported by findings from multiple studies [16,22–28] that report an association of R102G with AMD susceptibility, which are all among the Caucasian populations. In a most recent meta-analysis study of C3 risk to AMD involving 10,531 AMD individuals and 12,051 controls, the G allele of R102G was identified as a risk factor among Caucasians, but not in Asians [8], further supporting findings in this study.

Some limitations in the present study should be taken into consideration. Firstly, the relatively small number of samples could contribute to the lack of association and also the low frequency of the risk allele being studied. Secondly, the heterogeneity of patients, consisting of three multi-ethnics in Malaysia, could also affect the findings as different ethnicities might have different genetic profiles. Thirdly, there was no analysis of the C3 expression levels, which could be a risk factor for R102G. Moreover, there is the potential of nAMD being modified by the other genetic factors in the complement pathway apart from the C3 gene such as the *complement factor H (CFH)* and *complement factor 1 (CF1)* genes and other variants may play a role in pathogenesis of AMD in the Malaysian population.

In conclusion, there was no significant association between C3 R102G gene polymorphism and the risk of nAMD among Malaysians. A larger sample size with variable SNPs should be conducted in future studies to identify further risk-associated variants related to nAMD.

Conflict of interest

There is no conflict of interest.

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