Clinical study of the correlation between complement factor H polymorphism and age-related macular degeneration

H.T. Dong*, J.X. Zhang*, Q.M. Li and F.Z. Li

Department of Ophthalmology, First Affiliated Hospital of Zhengzhou University, Zhengzhou, China

*These authors contributed equally to this study.
Corresponding author: Q.M. Li
E-mail: donghongtao211@163.com

ABSTRACT. This study aimed to investigate the correlation between age-related macular degeneration (AMD) of the liver-kidney yin-deficiency type and complement factor H (*CFH*) polymorphism, and to determine whether the C allele of the T1277C (Y402H) variant is a risk factor for this condition. We performed a case-control investigation of 60 patients with liver-kidney yin-deficiency AMD and 60 normal control subjects. Peripheral blood was collected from each participant for DNA extraction. Following amplification by polymerase chain reaction, the DNA samples were sequenced, and polymorphism of the *CFH* gene was examined. Data were analyzed with the chi-square test, with $P < 0.05$ signifying statistical significance. The frequency of the C allele was significantly higher in the wet than in the dry AMD group ($P = 0.044$). In addition, the TC and CC genotypes were markedly more common in the former than in the control group ($P = 0.013$), and there

was a significant difference in the distribution of the T and C alleles between wet AMD patients and control subjects (P < 0.05). Based on this, we conclude that liver-kidney yin-deficiency AMD is associated with the C allele and TC and CC genotypes of the *CFH* Y402H polymorphism. Among patients with this condition, *CFH* genotypes were normally distributed. The principal *CFH* genotypes that induce liver-kidney yin-deficiency AMD are the mutant homozygote CC and heterozygote TC forms. Moreover, C allele carriers are at higher risk of developing this disease.

INTRODUCTION

Age-related macular degeneration (AMD) is also known as senile macular degeneration. In recent years, as the population of China has aged, AMD-associated morbidity has been rising consistently, gradually making AMD the eye disease of most concern (Apte, 2008; Schramm et al., 2014). Currently, treatment of wet AMD principally relies on glucocorticoids, photodynamic therapy, laser photocoagulation, intravitreal injection of anti-vascular endothelial growth factor drugs, and macular surgery (Delcourt et al., 2001; Mulder et al., 2010). Recently, research concerning AMD pathogenesis has developed rapidly, mainly owing to genomic approaches, providing new ideas and methods for inserting foreign genes into target cells (Michels and Rosenfeld, 2005).

CFH polymorphism is closely related to the development of AMD. As our knowledge of genetics and inflammatory immunity has progressed, many genes associated with AMD are gradually being discovered, such as *CFH*, complement component 2, complement factor B, apolipoprotein E, hemicentin 1, and low-density lipoprotein receptor-related protein 6 (Dikmetas et al., 2013). The influence of *CFH* in this regard has been widely recognized in the literature, with the *CFH* polymorphism being identified as a key pathogenic factor in AMD, and used successfully in genetic studies to generate models of this disease (Imai et al., 2010; Abbas and Azzazy, 2013). Variation in the *CFH* gene mainly manifests as the polymorphism Y402H (T1277C), in which a T-to-C substitution at position 1277 in exon 9 leads to a change at residue 402 in the translated sequence, from tyrosine to histidine (Y402H). The C allele, which is associated with high risk of AMD, is present in 50.0 to 57.3% of cases (Trouw et al., 2011; Zhong et al., 2011).

External factors can induce such mutations, thus causing *CFH* gene polymorphism, and consequently, decreased expression of the encoded protein. Therefore, variation in the *CFH* gene, consisting of polymorphism, is a key pathogenic factor in AMD. Using molecular biological methods, the present study revealed that *CFH* genotypes were distributed regularly among patients with liver-kidney yin-deficiency AMD, the mutant homozygote CC and heterozygote TC genotypes predominated in this group, *CFH* variation characterized by polymorphism was closely associated with AMD, and the C allele was a pathogenic factor conferring high risk of this disease. In brief, this study provides guidance for AMD research and treatment in the context of traditional Chinese medicine.

MATERIAL AND METHODS

General materials

We selected 60 liver-kidney yin-deficiency AMD patients (the test group, comprising 20 men and 40 women) having been treated in the ophthalmology and in-patient departments of the First Affiliated Hospital of Zhengzhou University from January 2013 to June 2015. Using routine examination procedures for this disease and tests of general health, these patients were found to conform to the inclusion criteria. In addition, we enrolled 60 healthy people (24 men and 36 women) for the control group. We then analyzed the characteristics of *CFH* gene variation with molecular biological techniques. Before participation, the patients and their families were informed of the research process and potential risks in detail, and their rights of informed consent were fully respected. In addition, voluntarily signed written informed consent was obtained from the patients' families and included in their medical records. This study was assessed by the medical research Ethics Committee of the First Affiliated Hospital of Zhengzhou University, and was in accordance with international and national ethics requirements for biomedical research. Therefore, the performance of this study was approved.

Diagnostic criteria

AMD diagnostic criteria

The diagnostic criteria for dry (atrophic) AMD were as follows: patients were over 55 years old; eyesight had weakened gradually; the fundus showed early macular depigmentation; the central light reflex was not clear; in the early phase, quasi-circular yellowish-white drusen were observed; in the late phase of lesions, pigment disorder, depigmentation, or geographic atrophy were seen; fundus fluorescein angiography (FFA) showed no fluorescein leakage or weak fluorescence in the macular area.

The diagnostic criteria for wet (exudative) AMD were as follows: patients were over 55 years old; eyesight had weakened rapidly; in the early phase, the fundus exhibited a grayish-yellow lesion in the macular area; the light reflex was not clear or was lost in the macula fovea centralis; drusen were present; edema was observed in the area of the lesion; during the intermediate phase, serous or hemorrhagic retinal pigment epithelial detachment in the macular area was seen; in more serious cases, subretinal, intraretinal, or vitreous hemorrhage was noted; scars formed in the late phase of lesions; FFA showed choroidal neovascularization in the macular area, fluorescent leakage, and blocked fluorescence caused by hemorrhage.

Control group criteria

Control subjects were at least 55 years old and were free of AMD and other eye diseases. In this study, we examined three recognized AMD risk factors, including smoking, body mass index (BMI), and blood pressure. The following were considered to increase risk: smoking at least one cigarette per day for more than 2 years; being overweight (BMI $\geq$ 23.0 kg/m^2); and having high blood pressure, defined as systolic pressure $\geq$160 mmHg, diastolic pressure $\geq$95 mmHg, and a history of high blood pressure.

Liver-kidney yin deficiency diagnostic criteria

According to the state standard, the diagnostic criteria for liver-kidney yin deficiency included thirst, dry throat, dysphoria in the chest, palms, and soles, dizziness, tinnitus, lumbar debility, insomnia, drowsiness, a red tongue with little coating, and a weak pulse.

AMD inclusion criteria

In the test group, patients met the diagnostic criteria of AMD and liver-kidney yin deficiency and gave their signed informed consent. Control subjects had no eye diseases. In both groups, ages ranged from 55 to 90 years.

Exclusion criteria

Reasons for exclusion included: pregnancy, lactation, abnormal liver function, a history of allergy, serious primary diseases of the heart, blood vessels, liver, kidney, or hematopoietic system, mental illness, poor compliance, inconvenience of regular examination or treatment, and refusal to sign informed consent.

Clinical examination indexes

Assessments of general health, including blood, urine, and stool examination, liver function testing, and an electrocardiogram were carried out. Routine examination for AMD was performed before inclusion of patients, using the following indexes and instruments: eyesight test, slit lamp, Amsler chart, ophthalmoscope, perimetry, FFA, and optical tomography.

CFH genotyping

Venous blood sampling of AMD patients and control subjects was performed to establish the distribution of the TT, TC, and CC genotypes and C allele of the *CFH* polymorphism.

Procedure

Firstly, genomic DNA was extracted. Fasting peripheral venous blood (2 mL) was collected using 2% ethylenediaminetetraacetic acid as an anticoagulant. Total genomic DNA was then isolated and preserved at -80°C.

Secondly, polymerase chain reaction (PCR) amplification in combination with restriction fragment length polymorphism (RFLP) was used for analysis of *CFH* sequence variations. The following primers targeting the *CFH* Y402H (rs1061170) region were synthesized: forward (10 μM), 5'-TCA TTG TTA TGG TCC TTA GGA AA-3'; and reverse (10 μM), 5'-TTA GAA AGA CAT GAA CAT GCT AGG-3'. The 10-μL PCR system comprised 1.0 μL 10X *Taq* buffer, 0.5 μL deoxynucleotides (2.5 mM), 0.1 μL each rs1061170 primer (50 μM), 0.06 μL DreamTaq (5 U/μL; Thermo Scientific, Waltham, MA, USA), 7.94 μL distilled H_2O, and 0.3 μL template DNA. Amplification conditions consisted of 95°C pre-denaturation for 5 min, and 40 cycles of 95°C denaturation for 30 s, 60°C annealing for 45 s, and 72°C

extension for 60 s. Alleles were detected using the restriction enzyme *Hin*1II. Enzyme digestion products were separated into different bands by 2% agarose gel electrophoresis alongside a DNA size marker, and following ethidium bromide staining, the results were observed under an ultraviolet lamp.

Thirdly, the fragments resulting from restriction enzyme digestion of the PCR product were analyzed. The total length of the amplified *CFH* Y402H (rs1061170)-containing sequence was 241 bp. After *Hin*1II digestion, the wild-type (TT), heterozygous (TC), and homozygous mutant (CC) fragments were identified. To verify these PCR-RFLP results, PCR products for each genotype were sequenced.

Statistical methods

Using the SPSS 19.0 statistical software (IBM Corp., Armonk, NY, USA), a database was created, into which results were logged. After checks and modifications, the database was converted to generate SPSS 19.0 data files. The correlation between liver-kidney yin-deficiency AMD and the TC and CC genotypes of *CFH* Y402H was analyzed with the chi-square test. This showed that the C allele was associated with a high risk of this disease.

RESULTS

General characteristics of the study population

In strict accordance with the inclusion and exclusion criteria, we selected 60 liver-kidney yin-deficiency AMD patients undergoing treatment in the ophthalmology and in-patient departments of the First Affiliated Hospital of Zhengzhou University from January 2013 to June 2015. Their average age was 66.7 ± 6.9 years (Table 1). Sixty healthy people were selected for the control group, with an average age of 66.2 ± 6.3 years. Differences in age between the test and control groups were not significant, with the *t*-test returning a P value of 0.521. There were 20 male and 40 female test subjects and 24 male and 36 female control participants. Using the chi-square test, no statistically significant difference in gender was detected between the two groups (P = 0.62). Neither smoking history (Table 2) nor BMI (Table 3) significantly differed between the groups (P > 0.05). However, high blood pressure (Table 4) was significantly more common among AMD patients than control subjects (P < 0.05).

Table 1. Age and gender distribution within the study population.

Age (years)	Test group			Control group		
	Male	Female	Total	Male	Female	Total
55-65	2	2	4	3	2	5
66-75	7	15	22	9	12	21
76-85	8	17	25	7	14	21
>85	3	6	9	5	8	13
Total	20	40	60	24	36	60

For age, chi-square = 2.268 and P = 0.521, indicating no statistically significant difference. For gender, chi-square = 0.248 and P = 0.619, indicating no statistically significant difference.

Table 2. Statistics regarding smoking status of the test and control subjects.

	Test group	Control group	Odds ratio (95%CI)	Chi-square	P
Smokers	28 (46.67%)	33 (55.00%)	1.128 (0.912-1.396)	1.205	0.273
Non-smokers	32 (53.33%)	27 (45.00%)	0.889 (0.711-1.098)		

CI = confidence interval.

Table 3. Statistics concerning body mass index in the test and control groups.

	Test group	Control group	Odds ratio (95%CI)	Chi-square	P
Higher than normal	21 (35.00%)	19 (31.67%)	1.097 (0.846-1.429)	0.496	0.479
Normal	39 (65.00%)	41 (68.33%)	0.937 (0.785-1.121)		

CI = confidence interval.

Table 4. Statistics concerning blood pressure of test and control subjects.

	Test group	Control group	Odds ratio (95%CI)	Chi-square	P
Normal	38 (63.33%)	44 (73.33%)	1.176 (1.008-1.368)	4.296	0.041
High blood pressure	22 (36.67)	16 (26.67%)	0.724 (0.531-0.992)		

CI = confidence interval.

PCR amplification

A 241-bp DNA band was amplified for each AMD patient and control subject (Figure 1). By comparison with a DNA size marker, the PCR product length was in accordance with expectations from the primer design.
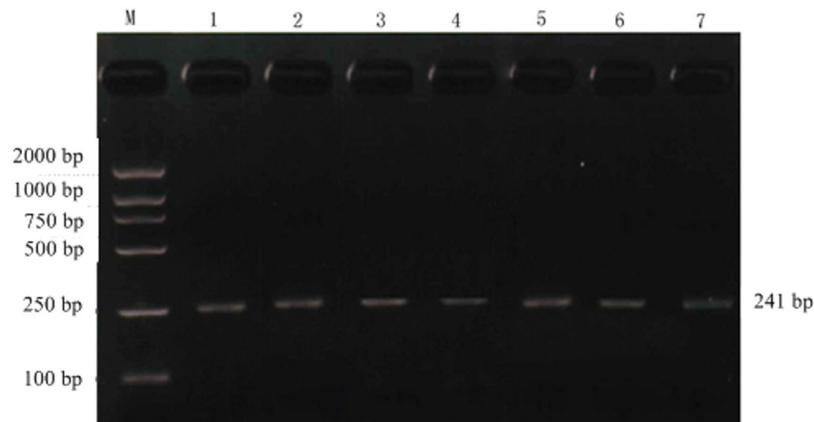
Figure 1. Agarose gel electrophoresis of the target gene polymerase chain reaction product. *Lane M* is a DL2000 marker; *lanes 1* to *7* contain amplified genomic DNA from collected samples.

The PCR product was digested with a restriction enzyme (Fujian Huacan Pharmaceutical Co., Ltd., Sanming City, Fujian Province, China), and 5 µL of this reaction mixture was subjected to 2% agarose gel electrophoresis, by which the genotype of each study subject could be accurately identified (Figure 2). Approximately 20% of the samples were

selected randomly for genotype verification by sequencing. The results were consistent with those obtained by restriction enzyme digestion (100% accuracy).
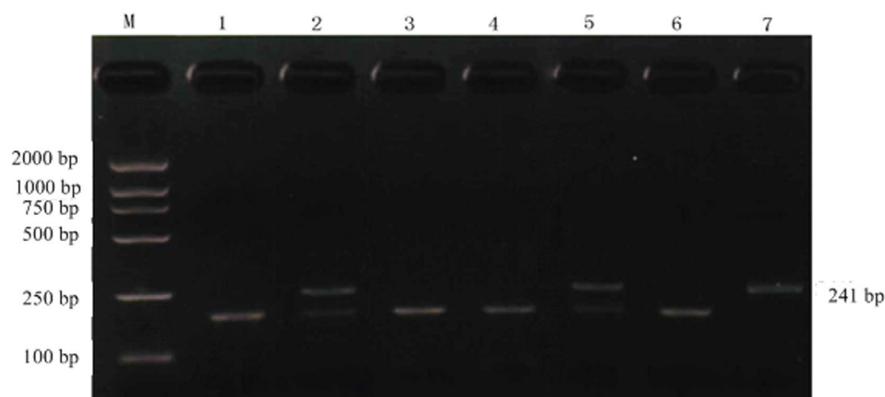
Figure 2. Restriction enzyme digestion typing of the Y402H *CFH* gene polymorphism. *Lane 1* is a DL2000 marker; *lanes 1*, *3*, *4*, and *6* show the TT genotype; *lanes 2* and *5*, the TC genotype; and *lane 7*, the CC genotype.

DNA sequencing results

PCR product sequencing successfully identified the T-to-C mutation at position 1277 in exon 9 of the *CFH* gene, leading codon 402 to encode histidine rather than tyrosine (Y402H). A T residue at this position was also detected in some samples, representing the wild-type sequence.

Correlation analysis of the Y402H *CFH* polymorphism and AMD

The distribution of Y402H alleles and genotypes in the control group was in accordance with the Hardy-Weinberg equilibrium. The allele and genotype frequencies of this polymorphism did not significantly differ between the overall test and control groups (P = 0.093 and 0.098, respectively), nor between the dry AMD and control groups (P = 0.724 and 0.711, respectively). However, the frequency of the C allele (P = 0.012) and TC and CC genotypes (P = 0.013) was markedly higher among wet AMD patients than control subjects. In addition, we compared the two AMD groups, finding that genotype frequencies were not significantly different among patients with the dry and wet forms of this disease (P = 0.052), although the C allele was significantly more common in latter than the former (P = 0.044). Statistical data are shown in Tables 5 and 6.

Table 5. Statistical analysis of allele frequencies in age-related macular degeneration (AMD) and control groups.

Group	Allele		Chi-square	P	OR (95%CI)	Chi-square	P
	C (%)	T (%)					
Control	46 (76.70%)	14 (23.30%)	-	-	-	-	-
Dry AMD	3 (5.00%)	12 (20.00%)	0.126	0.724	1.133 (0.574-2.221)	3.984	0.044
Wet AMD	9 (15.00%)	36 (60.00%)	6.577	0.012	2.215 (1.193-4.114)		
Overall AMD	12 (20.00%)	48 (80.00%)	2.832	0.093	1.603 (0.923-2.781)		

OR = odds ratio, CI = confidence interval.

Table 6. Statistical analysis of genotype frequencies in age-related macular degeneration (AMD) and control groups.

Group	Genotype			HWE	Chi-square	P	OR (95%CI)	Chi-square	P
	TT (%)	TC (%)	CC (%)						
Control	38 (63.33)	16 (26.67)	6 (10.00)	0.34	-	-	-	-	-
Dry AMD	4 (6.67)	8 (13.33)	36 (60.00)	0.40	0.137	0.711*	1.142 (0.564-2.313)	3.798	0.052
Wet AMD	2 (3.33)	4 (6.67)	6 (10.00)	0.45	6.318	0.013*	2.315 (0.790-4.504)		
Overall AMD	6 (10.00)	12 (20.00)	42 (70.00)	0.36	2.751	0.098*	1.631 (0.911-2.912)		

*TC and CC genotype frequencies were merged before comparison when the latter was low. OR = odds ratio, CI = confidence interval.

DISCUSSION

In this study, we identified a significant correlation between AMD pathogenesis and high blood pressure, but not smoking or BMI. Moreover, the Y402H polymorphism of the *CFH* gene was significantly associated with wet, but not dry, AMD. Taking into account current academic research, the etiology and pathogenesis of this disease remain unclear, yet an in-depth understanding of AMD risk factors is an important prerequisite for its prevention and treatment. Age is well recognized as a major contributor to AMD susceptibility, as is smoking (Clemons et al., 2005). Although the literature is not always consistent on this subject, it has been speculated that smoking may triple the risk of developing AMD (Buch, 2005) by affecting the retinal pigment epithelium, thus increasing oxidative stress and compromising microcirculation in the choroid blood vessels. In this study, smoking history was not significantly related to AMD pathogenesis, perhaps owing to the limited sample size. Due to the limitation of experimental instruments, we did not statistically assess other risk factors, such as oxidant intake and sun exposure. In addition to these environmental influences, systemic diseases or changes in body indexes (such as elevated BMI and C-reactive protein levels) may also be risk factors for eye diseases (Kaushik et al., 2008; van de Ven et al., 2012).

The role of genetic factors in the pathogenesis of AMD is a current focus of research. A related investigation found that the *CFH* Y402H polymorphism is significantly linked with susceptibility to wet AMD (Soubrane et al., 2003). In recent years, a number of research groups in North America have demonstrated that AMD risk is higher for carriers of this variant, and this has been confirmed by researchers in Finland, Italy, and France (Axer-Siegel et al., 2004; Shyong et al., 2005). In a Japanese study (Tanaka et al., 2011), the frequency of Y402H was found to be 4% in AMD and control groups, and two *CFH* haplotypes (IVS6 and Q672Q) were identified as AMD risk factors. In addition, a further haplotype (I62V) was reported to exert a favorable effect in AMD patients. Thus, we can conclude that the *CFH* polymorphism is related to this condition's pathogenesis, yet there have been no reports of this in China. In the present study, among the 60 AMD patients, 6 (10%), 12 (20%), and 42 (70%) carried the TT, TC, and CC genotypes, respectively. Of the 60 healthy controls, these figures were 38 (63.3%), 16 (26.7%), and 6 (10%), respectively. Comparing the AMD and control groups, statistical analysis revealed a significant difference in *CFH* polymorphism genotype frequencies (P < 0.05). The C and T alleles were present in 80 and 20% of the AMD patients and 76.7 and 23.3% of the control subjects, respectively. Accordingly, the distribution of these alleles significantly differed between wet AMD and control groups (P < 0.05). Therefore, liver-kidney yin-deficiency AMD is associated with the C allele and TC and CC genotypes of *CFH* Y402H. Among patients with this disease, *CFH* genotypes were normally distributed,

and the mutant homozygote CC and heterozygote TC forms predominated. Variation in the *CFH* gene is obviously connected with AMD, the T-to-C base change being a key variant in this relationship (Hooper et al., 2008; Biswas et al., 2011). Ages of patients with liver-kidney yin-deficiency AMD and control subjects were normally distributed, and according to the *t*-test, did not significantly differ ($P > 0.05$). In addition, based on the chi-square test, these groups showed no difference in terms of gender ($P > 0.05$).

As the main inhibitor of the complement system, the CFH protein plays an important role in complement activation. CFH can combine with C3b, inhibiting the formation of alternative-pathway C3 convertase, regulating and controlling the extent of complement activation (Yücel et al., 2012). Environmental factors such as obesity and smoking can interact with the *CFH* gene polymorphism, leading to reduced CFH expression and activation of the complement system, and therefore significantly increasing the concentrations of C5b-9 (membrane attack complex) and C3b (Atmaca et al., 2011; Schmitz-Valckenberg et al., 2011; Cruz-Guilloty et al., 2014). C5b-9 is deposited on the basement membrane of retinal pigment epithelium (RPE) cells, accelerating the formation of drusen. Deposition also occurs between the RPE and Bruch's membrane, resulting in degeneration of the RPE-Bruch's membrane-choriocapillaris complex, as well as atrophy of the macular area and posterior pole (Seddon et al., 2007). Furthermore, the collagen within Bruch's membrane thickens, and fracturing of its elastic fiber layer accelerates. The choriocapillaris enters the retinal neuroepithelium layer and RPE, resulting in subretinal neovascularization, aggravating AMD (Smailhodzic et al., 2012).

In this study, different *CFH* genotypes were analyzed. The pathogenesis of liver-kidney yin-deficiency AMD was found to be associated with the T-to-C polymorphism at position 1277 of the *CFH* gene. Among patients with this condition, *CFH* genotypes were distributed regularly and the mutant homozygote CC and heterozygote TC genotypes predominated. The C allele was observed to be a pathogenic factor conferring high risk of liver-kidney yin-deficiency AMD. Moreover, this disease was not determined to be significantly related to age or gender in either of the study groups.

Conflicts of interest

The authors declare no conflict of interest.

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