Association of Apolipoprotein E Alleles with Susceptibility to Age-Related Macular Degeneration in Iranian Patients

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Abstract

Background: This study aimed at investigating the association between alleles and genotypes of *APOE* and developing Age-related macular degeneration (AMD).

Materials and Methods: After ophthalmological examination, 120 patients with confirmed AMD and 120 healthy controls were enrolled. The polymorphic segment of *APOE* gene was PCR-amplified and sequenced to determine the frequency distribution of polymorphic alleles and genotypes of this gene in sample population.

Results: The frequency distribution of *APOE* alleles and genotypes differed significantly between the patients and control groups ($P<0.05$). The frequency of *APOE*ε2 was higher in patients than that of the controls ($P = 0.00$) and this variant allele showed a significant association with AMD even after removal of the effects of age, sex and smoking in logistic regression analysis ($P = 0.00$, OR= 3.439; CI 95% 1.664-7.108). On the other hand, the frequency distribution of *APOE*ε4 was not statistically different between patients and healthy controls.

Conclusion: The results showed a moderate positive association between *APOE* ε2 and AMD, but no specific role was found for *APOE* ε4 in protection against AMD. However, more studies are required to clarify the possible role of *APOE* in the pathogenesis of AMD.

Keywords: Age-related macular degeneration; Association study; Apolipoprotein E; Vision

Introduction

Age-related macular degeneration (AMD) is a multifactorial disease, which is known to be the main cause of visual impairment in the elderly and accounts for nearly 50% of all new blindness cases in industrialized world. The public health burdens of AMDtend to increase since the senile populations begin to outnumber young in many countries (1-4). The etiopathology of AMD is not very well defined yet. However, it is believed that it is a multifactorial disease resulting from interaction of genetic, environmental and demographic factors (5, 6). As an example, smoking increases the risk of development of this disease for two to four times. But, it was noted that contribution of genetic factors in pathogenesis of AMD is higher than other factors (7, 8).

Due to the importance of their contribution in AMD, there are three major susceptibility loci of 1q31, 10q26, and 19q13.2. Of these loci, 1q31 and 10q26

are to gather responsible for more than half of all AMD cases. Variants of *CFH* gene located on 1q31 locus and other complement pathway gene (e.g. *C2/CHB*, *C3* and *CFI*) variants are strongly associated with AMD and so, an inflammatory process is thought to be involved in pathogenesis of AMD (9-15). A linkage disequilibrium analysis between 10q26 genes and a recently identified functional variant of age-related maculopathy susceptibility 2 genes (*ARMS2*) indicated that *ARMS2* could be associated with AMD. Candidate gene studies targeted 19q13.2, because of apolipoprotein E gene (*APOE*) (16-20).

ApoE serves as a common apolipoprotein and its supportive and protective roles in neural tissues make it a unique molecule and so, it has been suggested that *APOE* may be related to neurodegenerative conditions.

Apo E plays a pivotal role in lipid transportation and is known to be one of the drusen making elements (21). *APOE* is a polymorphic gene and makes a large number of different apo E isoforms of which the apoE2, apoE3 and apoE4, encoded by ε2, ε3 and ε4 allelic variants are the main isoforms identified by isoelectric focusing. Apo E molecules have 299 amino acids in length. The isoforms differ from one another in two amino acid moieties of residues 112 and 158, which in apo E2, apo E3 and apo E4 include cystein/cystein, cystein/arginine and arginine/arginine ,respectively (22, 23). Genetic studies showed that *APOE* variants are associated with increased risk of Alzheimer's disease and AMD (16, 21).

Among different alleles, *APOE*ε3 is considered to be the wild-type or ancestral whose frequency has been reported to be as high as 85% in general population. ε2 and ε4 are believed to be the variant froms of this allele with reported frequencies of up to 5 and 10% in general population (22). Some studies have suggested that, ε2 increases the risk of development of AMD (7, 24) and ε4 confers a protection for this disease (16, 21, 24, 25). However, the same results have not been obtained by others who reported lack of association between apo E variants and AMD (26).

Considering such contradictory results, we aimed to investigate the association between variants of *APOE* and AMD by a genetic association study in an Iranian population.

Materials and Methods
Study population
The subjects were recruited from the Medical Genetics Laboratory of Fazeli-Sanati in Tehran, between 2009-2011 and included 120 unrelated AMD patients (71 men, 49 women; mean age 71.9 ± 7.9 years old) and 120 age and sex-matched healthy controls (71 men, 49 women; mean age 71.6 ± 6.0 years old). Demographic data were obtained through standard questionnaires. All subjects had undergone a complete ophthalmologic examination and their diagnosis was confirmed by ophthalmologist. Individuals with good visual acuity and no signs of macular abnormalities including drusen, exudative changes, pigmentary alterations and diabetic maculopathy have been considered as healthy controls. The diagnosis of AMD has been made based on international classification and grading system for age-related maculopathy and age-related macular degeneration (27). AMD patients were classified into wet and dry groups and those concurrently affected by any other types of retinal disorders were excluded from the study.

Before enrolling in this study, all subjects signed a written informed consent.

APOE genotyping
Venous blood samples (5ml) were collected in EDTA-containing tubes and stored in −80oC until the time of DNA extraction. Genomic DNA was isolated from the whole blood leukocytes by GeneJETTM Genomic DNA Purification Kit (Fermentas, Lithonia) according to the manufacturer's protocol. A segment of *APOE* gene containing single nucleotide polymorphic positions (273 length in base pairs), from amino acid 112 (rs429358) to 158 (rs7412), was PCR-amplified using specific primers (26) and Cinna Gen Taq DNA Polymerase kit (SinaClon, Iran) according to manufecturere's instructions. The PCR cycling profile began with a denaturation step (95°C for 5 min), followed by 30 cycles of amplification (95 °C for 20 s, 62 °C for 30 s, and 72 °C for 35 s) and a cycle of final extension (72 °C for 10 min). To confirm PCR amplification, 5ul of each PCR products were analyzed by electrophoresis in 2% agarose. A total of 15ul of PCR products were reserved for direct sequencing.

To identify the allelic variants in each sample, PCR products were sequenced using ABI 3130 Sequencer (Applied Biosystems, USA). The results were then aligned against the *APOE* gene reference sequence in GenBank database (Gene ID 384).

Statistical Analysis
SPSS (Version 18.0; SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Distribution of the genotype was checked to be in Hardy-Weinberg equilibrium. To compare qualitative and quantitative variables between the groups, Chi-square, Fisher's exact test, and student's t test were applied. A logistic regression analysis was performed to adjust the risk of polymorphism for the effects of age, sex, and smoking. A P -value of less than 0.05 was considered to be significant.

Ethics Statement
The study adhered to the principles of the Declaration of Helsinki and was approved by the Tehran University of Medical Sciences Ethics Committee.

Results
Table 1 shows demographic characteristics of the study population. Genotype distribution had no deviation from Hardy-Weinberg equilibrium (P = 0.55).

Table 1. Characteristics of study population.

Parameter	Controls (n = 120)	Patients (n = 120)	P-value
Sex (male/female)	71/49	71/49	1.00
Age (year)	71.6 ± 6.0	71.9 ± 7.9	0.68
Smoking[1] (yes/no)	16/104	24/96	0.22
High blood pressure[2] (yes/no)	49/71	32/88	0.20
Family history (yes/no)	0/120	/112	0.00

1-Individuals smoking more than 5 cigarettes per day were considered smoker.
2- Individuals with blood pressure higher than 90/140 mmHg were considered to have high blood pressure.

APOE allele and genotype distributions differed significantly between patients and the control group (table 2). The frequency distribution of APOE allele in healthy controls was similar to another report from Iran (28).

To analyze the effects of ε2 allele in pathogenesis and ε4 allele in protection from AMD, the study population was independently grouped into ε2+ (individuals with ε2ε2, ε2ε3 and ε2ε4 genotype), ε2- (individuals with ε3ε3, ε3ε4 and ε4ε4 genotype), and ε4+ and ε4- groups. We observed the frequency of ε2-containing genotype to be significantly higher in patients compared with that of the controls (P = 0.00, OR=3.27; CI 95%= 1.59-6.72). While the same analysis for ε4-containing genotypes did not show any significant difference between patients and controls (P = 0.84, OR=0.86; CI 95%= 0.39-1.8) (table 3).

The allelic and genotype distributions of dry and wet AMD subgroups were not significantly different (P = 0.02). There was no association between smoking and AMD development (P = 0.22). Logistic regression analysis demonstrated that even after the removal of the effects of age, sex and smoking, allele ε2 exhibited a moderate positive association with AMD (P = 0.00, OR= 3.439; CI 95% 1.664-7.108).

Discussion
This study showed a statistically significant association between the APOEε2 allele and development of AMD in Iranian subjects. This relationship has also been reported in Australian (29), and Italian (24) populations but this association is not always reproducible. Several other studies reported no association between APOE and AMD in Chinese, Japanese and Caucasian (26, 30-32). We have shown that a ε2+ genotype increases the risk of development of AMD. The distribution of genotypes in our sample was in concordance with another Iranian population study on APOE genotyping in which subjects were recruited from a medical center in Tehran (7). Although all participants in this study were Iranians, they belonged to different Iranian ethnic groups; therefore, our results should be interpreted carefully.

Table 2. Allele and genotype distribution in study population.

Allele/Genotype	Controls (n = 120)	Patients (n = 120)	P-value
Allele			
ε2	13 (5.41%)	39 (16.25%)	
ε3	211 (87.91%)	187 (77.91%)	0.00
ε4	16 (6.66%)	14 (5.83%)	
Genotype			
ε2 ε2	1 (0.83%)	7 (5.83%)	
ε2 ε3	10 (8.33%)	23 (19.16%)	
ε2 ε4	1 (0.83%)	2 (1.66%)	
ε3 ε3	93 (77.50%)	76 (63.33%)	0.01
ε3 ε4	15 (12.50%)	12 (10.00%)	
ε4 ε4	0 (0.00%)	0 (0.00%)	

The underlying mechanism of contribution of APOE gene variation with pathogenesis of AMD is not very well understood yet. But, as drusen is formed by cell membrane deposits (lipids) and apolipo proteins (Apo E and B), it could be hypothesized that the variant allele affects the lipid metabolism and so contributes to the pathogenesis of this disease. In fact, since ε2, ε3, and ε4 alleles encode a segment in apo E receptor-binding region, they affect the receptor-binding affinity of this molecule (25). It is known that the ε2 has a Cys at positions 112 and 158, which decrease the receptor-binding affinity of this molecule to its lowest. Therefore, the lipids, which are released through the age-related physiological processes in eye, could not be effectively transported by the apo E and this overall process may predispose the individual to the macular degeneration (25). In addition to what discussed above on different

biochemical characteristic of APOE isoforms, the genetic architecture of ε2 and ε4 alleles may also explain their opposite roles in predisposing individuals to the AMD. The ε2 and ε4 allele would be defined by "TT" and "CC" allele combinations of rs429358 (C/T) and rs7412 (C/T) on the same chromosome, respectively. For the rs429358, "C" is ancestral allele and "T" is derived allele. It had been reported that derived alleles predispose individuals to

diseases more than ancestral alleles do (33). This fact corroborates predisposing role of ε2 allele (harboring rs429358 T) for AMD. On the other hand, alleles existing longer in population (C for rs429358) tend to show weaker linkage disequilibrium with neighboring alleles, including the causal alleles, and are less likely to link with a disease associated-SNP (33). Protective role of rs429358 C in AMD can be deduced from this fact.

Table 3. Frequency of ε2 and ε4 in patients and controls.

	Frequencies of genotype groups (number)				
	ε2+	ε2-	ε4+	ε4-	*P* value
Controls	12 (9.99%)	118 (90.10%)	16 (13.3%)	94 (78.3%)	
Patients	32 (27%)	88 (73%)	14 (12.0%)	106 (88.0%)	0.00

In contrast with reports that concluded sex-specific effects of *APOE* (29, 34), our results show that there is no gender-specific effect for ε2 and both sexes have the same risk of development of this disease. On the other hand, although several studies have shown that *APOE* ε4 allele protects against development of AMD (16, 21, 24), our results do not provide any suggestive evidence for this protection. This could be due to the low number of individuals with this variant in our sample. Indeed, to investigate *APOE* ε4 allele association with AMD, studies with larger sample size are necessary. A pooled analysis of 15 studies (with 10544 affected and 10623 unaffected individuals) could fairly reveal that *APOE* ε4 allele associated with late onset AMD (OR=0.72; CI: 0.65–0.74; P=4.41×10−11).

As a drawback, there were limited numbers of AMD cases with the history of familial disease in our work. Therefore, it was not plausible to conclude any remarks on familial AMD based on our current data.

In conclusion, our results suggest a statistically significant association (P= 0.00) between *APOE* ε2 gene variant and development of AMD in Iranian subjects. However, further studies are required to reveal the possible role of *APOE* in pathogenesis of AMD pathogenesis. Also, it would be interesting to study the linkage disequilibrium between *APOE* ε2 and the adjacent gene variants, which may lead to introduction of other genes in pathogenesis of AMD and reveal new pathologic mechanisms.

Authors' Contributions
MA, KM, and ARN carried out the design and coordinated the study, participated in most of the experiments and prepared the manuscript. HA, HG, AI, KGF and HN provided assistance in the design of the study, collecting patient data and samples and carried out most of the experiments. MM, AA, MHS and JA provided assistance for all experiments, statistical analysis and manuscript preparation. HG and MA and JA drafting of the manuscript.

Conflicts of Interest
The authors declare that there are no conflicts of interest.

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