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STUDY OF POLYMORPHISMS IN *CFH, ARMS2* AND *HTRA1* GENES AS POTENTIAL RISK FACTORS FOR AGE RELATED MACULAR DEGENERATION IN INDIAN PATIENTS

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Abstract

Background: Age-related macular degeneration (AMD) is a common disease in which both environmental and genetic factors have been implicated. Various single nucleotide polymorphisms (SNPs) have been found to be associated with AMD.

Aim: To study the association of polymorphisms in *CFH*, *ARMS2* and *HTRA1* genes with AMD in Indian patients.

Settings and Design: Case-control study

Methods and Material: Genotyping for *CFH* (Y402H), *ARMS2* (A69S), *HTRA1* (promoter region G/A) were performed in 121 AMD patients and 100 controls by PCR-RFLP.

Statistical Analysis Used: Hardy-Weinberg estimates (HWE) were calculated. Analysis for genotypes was done under additive, recessive as well as dominant models of inheritance showing odds of occurrence using Fisher's exact test. Pairwise linkage disequilibrium (LD) was measured using SNP Stats to predict Lewontin's LD (D') and correlation coefficient (r).

Results: Genotype analysis of all three polymorphisms (Y402H in *CFH*, A69S in *ARMS2* and G/A in *HTRA1* promoter region) showed the strongest association of AMD with Y402H polymorphism in *CFH* gene. Odds ratio for Y402H allele in *CFH* gene was 2.66 under recessive model. Odds ratio for polymorphisms in *ARMS2* and *HTRA1* genes were 2.41 and 1.97 respectively. Significant LD (D'=0.87, r=0.81) was observed in the presence of variant alleles *HTRA1A* and *ARMS2T* for AMD.

Conclusions: Present study suggests that all the three polymorphisms *CFH* Y402H (T/C), *ARMS2* A69S (G/T) and *HTRA1* promoter region (G/A) alleles are potential risk factors and are independently associated with AMD in Indian population. Detection of individuals at risk could lead to strategies for prevention, early diagnosis and management of AMD.

Key Words: AMD; *ARMS2*; *CFH*; *HTRA1*; Indian; Polymorphism; PCR-RFLP

INTRODUCTION

Age related macular degeneration (AMD), also referred to as age related maculopathy, is a degenerative disease of the retina that causes progressive impairment of central vision and is the leading cause of irreversible vision loss in elderly people. The prevalence of the disease increases with age, affecting 9% of the population over age 65 years and 28% over age 75 years[1]. The pathologic hallmark of the disease is drusen, deposits of protein and lipid in the retinal pigment epithelium or Bruch's membrane. The maculopathy progresses to degeneration in two forms: geographic atrophy, in which there is loss of retinal pigment epithelium and photoreceptors and neovascular AMD, in which there is choroidal neovascularization and hemorrhages.

AMD is considered to be a multifactorial disease. Numerous studies indicate that risk factors include age, gender, ethnicity, smoking, hypertension, diet and also genetic factors due to familial aggregations[2]. Twin and segregation analysis studies suggest a significant genetic contribution to the disease[3,4]. Whole genome scans have led to identification of the association of AMD with chromosomal loci 1q, 9q, 10q, 16q and 22q by linkage analysis[5-7]. Complement factor H (*CFH*) gene on chromosome 1q32 has been determined to be strongly associated with AMD[7-10]. *CFH* Y402H (tyrosine to histidine substitution at amino acid position 402 in the *CFH* protein) is the result of T/C transition at nucleotide position 1277 in exon-9 of the gene and is the first major susceptibility allele for AMD reported in the literature. The CFH gene encodes complement factor H which is a serum glycoprotein that regulates the function of the alternative complement pathway. It binds to C3b, accelerates the decay of the alternative pathway convertase C3bBb, and also acts as a cofactor for another C3b inhibitor i.e. complement factor I[11, 12]. The Y402H polymorphism is in a region of CFH that binds heparin and C-reactive protein. CFH and its ligand C3b/iC3b have been found to co-localize in amyloid-containing sub-structural elements within macular

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drusen from patients with AMD and studies have suggested that variations within the CFH gene may attenuate complement inhibitory function and put retinal pigmented epithelial and choroidal cells at risk for alternative pathway-mediated complement attack[13].

Previous studies have also shown evidence of association of AMD with loci at 10q26 which overlaps with three genes, namely: *PLEKHA1*, LOC387715/*ARMS2* (age-related maculopathy susceptibility 2), and *HTRA1* (high-temperature requirement factor A1)[14, 15]. *PLEKHA1* has a pleckstrin homology domain in exon 12, whereas LOC387715/*ARMS2* encodes a protein of unknown function and it spans a 200-kb region[16-18]. Polymorphism G/T (rs10490924) at codon 69 (changes Alanine to Serine- A69S) found on chromosome 10q26 at the exon 1 of *ARMS2* is an important polymorphism showing association with AMD. The normal protein encoded by the *ARMS2* gene has been shown to localize most prominently to mitochondria in the ellipsoid region of the rod and cone segments, and therefore it has been proposed that the gene is possibly involved in the pathogenesis of age-related macular degeneration through mitochondria-related pathways[19, 20].

HTRA1 (high-temperature requirement factor A1) gene which encodes the heat shock serine protease is also located on chromosome 10q26 and has been found to be associated with AMD. A polymorphism G/A in the promoter of the *HTRA1* gene (rs11200638) was found to be associated with AMD risk[21, 22]. Limited studies available from India have shown association with *CFH, ARMS2* and *HTRA1* polymorphisms[23, 24].

MATERIALS AND METHODS

The study protocol was approved by the Institutional Ethics Committee. All participants received a standard ophthalmological examination, including visual acuity measurement, slit-lamp biomicroscopy and dilated fundus examination performed by a retinal specialist. The diagnosis was confirmed in fluorescein angiography and cases with both dry and wet types of AMD were included. Controls were more than 70 years of age and were confirmed not to have clinical evidence of AMD in any of the eyes by ophthalmological examination. Sample size was calculated before the study with the use of Quanto software version 1.2 by the institute statistician. The variables set were as follows- minimum allele frequency of gene and environment effect 0.30, relative risk 2, prevalence 0.1% and type one error (alpha) as 0.05. For the 80% power of the study, the sample size was calculated to be 92. Samples were collected after obtaining informed written consent from 121 clinically diagnosed AMD cases and 100 controls. There is no conflict of interest.

Genomic DNA was extracted from the peripheral blood of each individual using a DNA extraction and purification kit (Qiagen Blood DNA Mini Kit, Hilden, Germany), according to the manufacturer's instructions. Genotyping was performed by polymerase chain reaction and restriction fragment length polymorphism method (PCR-RFLP). The reaction mixture common to all three genotypes consisted of: 10X buffer with $MgCl_2$ 2.5 µl, Taq 1 U/µl- 1.5µl, dNTP 10 milimoles - 1µl, forward primer 10 picomoles-1µl, Reverse primer 10 picomoles - 1µl, DNA – 1.5µl (50 ng/µl) and HPLC water 16.5 µl per 25µl of the reaction mixture. Details of the primers and the restriction digestion reactions used for the study of each of the polymorphisms are given in Table I[25-27]. PCR conditions were as follows: hot lid 105°C for 5 min, denaturation 95°C for 5 min, 35 cycles consisting of denaturation at 94°C for 45 seconds, annealing for 45 seconds (at 50.7°C for CFH, at 65.1°C for ARMS2 and at 62.5°C for HTRA1) and extension at 72 °C for 1.5 minutes, followed by final extension at 72°C for 5 minutes and hold at 15°C forever. Aliquots of amplified uncut PCR products were resolved by electrophoresis in 2% agarose gels with 0.5µg/ml ethidium bromide and visualized under UV illuminator. Samples were then used for RFLP (restriction fragment length polymorphism). For *CFH* gene PCR product (~442 bp) was digested with 2 units of *Nla*III (New England Biolabs, Ipswich, Massachusetts), for *ARMS2* gene PCR product (158 bp) was digested with 2 units of *Fnu*4HI (New England Biolabs) and for *HTRA1* gene PCR product 208 bp was digested with 2 units of *Msp*I (New England Biolabs), incubated at 37°C overnight. Gel run using polyacrylamide gel electrophoresis (PAGE) was stained with ethidium bromide. Images of the gel (PAGE) were taken with a Molecular Imager Gel Doc XR System (Bio-Rad, Hercules, California). Genotypes were determined based on the restriction patterns. Ten percent of samples were subjected to sequencing and genotypes obtained by RFLP were confirmed by sequencing (Supplementary figure 1-3).

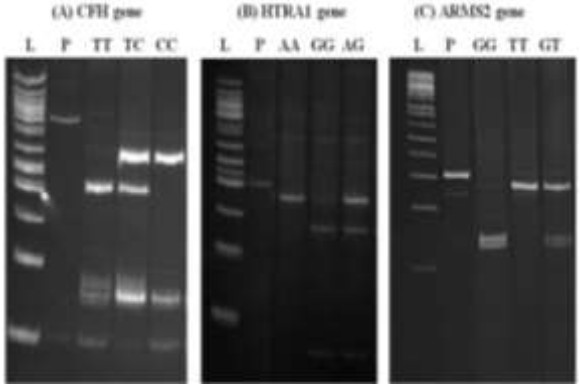

Figure 1: Gel Pictures of *CFH*, *HTRA1* and *ARMS2* genes RFLP analysis: In all of three figures 1st lane shows 50bp ladder (L) and 2nd lane shows undigested PCR product (P). Details of the digested product are mention in Table I.

Table I: Primer sequences, Restriction enzymes, and Fragment sizes

GENE & POLYMORPHISM	PRIMER SEQUENCE	PCR PRODUCT SIZE	ENZYME & SIZES OF FRAGMENT AFTER DIGESTION
CFH-Y402H [rs1061170] T>C	5'CTTTGTTAGTAACTTTAGTTCGTCTT|AG3' 5'ACAAGGTGACATAAACATTTTGCC-3'	442 bp	*Nla* III **Wild (T)**- 289+74+73+6bp **Mutant (C)**- 203+86+74+73+6bp
HTRA1 [rs11200638] G>A	5'- CGCATGCCACCCACAACAAC-3' 5'-GGTCACGCGCTGGTTCTG-3'	208 bp	*Msp* I **Wild (G)**- 141+44+23bp **Mutant (A)**- 185+23bp
ARMS2 Or LOC387715 [rs10490924] G>T	5'GTGGAGAAGGAGCCAGTGAC3' 5'CAGTTGTCAGGTGGTGCTGAG3'	158 bp	*Fnu*4 HI **Wild (G)**- 64+71+23bp **Mutant (T)**- 135+23bp

Allele and genotype frequencies were estimated by the allele counting method. Hardy-Weinberg estimates (HWE) for genotype frequencies were calculated using the Courtlab-HW calculator software. HWE was tested using the $\chi 2$ test with 1 degree of freedom. *p*-value $\leq$ 0.05 was considered statistically significant. Analysis for the genotypes was done under additive, recessive as well as dominant models of inheritance. Odds ratios were calculated and an estimation of confidence interval (95%) was made using Fisher's exact test. Statistical significance was accorded when p $\leq$ 0.05. All these calculations were carried out in commercial statistical analysis software (GraphPad Software ver. 3.05; GraphPad, San Diego, USA). Pairwise linkage disequilibrium (LD) was measured using SNP Stats to predict Lewontin's LD (D') and correlation coefficient (r).

RESULTS

A total of 121 patients with AMD (male-74 & female-47) and 100 controls (male-71 & female- 29) without AMD were recruited after detailed ophthalmological evaluation. The mean age was 70.40 (60-96) years for AMD patients and 74.42 (70- 95) years for controls. The studied controls were in Hardy-Weinberg equilibrium (Supplementary Table 1). Genotype data, for SNPs rs1061170: T>C in *CFH* (Y402H), rs10490924: G>T (A69S) in LOC387715/*ARMS2*, and rs11200638: G>A in the promoter region of *HTRA1* gene are summarized in Table II (Fig.1). Odds ratio under dominant model, of *CFH* gene (mutant homozygotes and heterozygotes as compared to the homozygous wild TT genotype) was 1.85 (95% CI: 1.05 – 3.24) with *p*-value of 0.033. The risk C allele frequencies were 117 (48.34%) in AMD cases and 69 (34.5%) in controls. Odds ratio was 1.777 (95% CI: 1.20 – 2.61; *p*-value 0.003). The AMD risk under recessive model in those who carried CC genotype compared with those who carried TT genotype or CT was 2.66 fold (95% CI: 1.26-5.64) and *p*-value was 0.009.

An association with AMD was also detected for SNP rs10490924: G>T polymorphism in LOC387715/*ARMS2* (Table II). The risk T allele frequencies were 139 (57.44%) in AMD cases and 87 (43.5%) in control samples, giving the odds ratio of 1.75 (95% CI: 1.20-2.55: *p*-value 0.004). Genotype distributions between AMD cases and controls were statistically significant. Compared to the wild type GG genotype under dominant model, odds ratio was 1.92 (95% CI: 1.02-3.62) (*p*-value 0.055). The odds ratio under recessive model for the mutant homozygous TT genotype was 2.41 (95% CI: 1.26-4.59 and *p*-value 0.008).

The SNP rs11200638: G>A in the promoter of *HTRA1* was also significantly associated with AMD (Table II). Frequencies of the risk A allele were 154 (63.64%) in AMD cases and 103 (51.5%) in controls, *p*-value was 0.010 and the odds ratio 1.64 (95% CI: 1.12 – 2.41). Genotype distribution of the *HTRA1* promoter polymorphism between AMD cases and controls was statistically significant. As compared to the wild type homozygote GG genotype under dominant model, odds ratio was 1.99 (95% CI: 0.97 - 4.08; *p*-value 0.07) and that for homozygous mutant AA genotype was odds ratio was 1.97 (95% CI; 1.10 - 3.52; *p*-value 0.02).

When the analysis was done for presence of any of the mutant alleles, the odds ratio was 3.919 and *p*-value was 0.060. In presence of 4, 5 or 6 mutant alleles the odds ratios were 4.192, 7.845 and 7.285 (*p*-value .063, .013 and .042) respectively. Though the number of patients with more than 3 alleles was very small, the data suggests that as the presence of more number of mutant alleles increases the odds ratio also increased.

Further the pairwise linkage disequilibrium (LD) analysis was carried out to estimate the Lewontin's LD measure (D') and correlation coefficient using Cramer's V statistic (r) (Table III). The LD analysis revealed Lewontin's D values > 0 among the studied mutant *ARMS2 and HTRA1* alleles suggesting involvement of multiple alleles in AMD progression and causation (Table III). The pairwise analysis revealed *ARMS2T-ARMS2G* (*D'*=0.99, r=0.97), *HTRA1G-ARMS2G* (*D'*=0.88, r=0.82), *HTRA1G-ARMS2T* (*D'*=0.92, r=0.83), *HTRA1A-ARMS2T* (*D'*=0.87, r=0.81) alleles to be in positive LD and justified by the significant *r* values.

Table II: Genotype frequencies for *CFH* T>C, LOC 387715 (*ARMS2*) G>T and *HTRA1* G>A polymorphisms in 121 AMD patients vs 100 controls

		AMD patients (N=121)	Controls (N=100)	OR	95% CI	p-value
***CFH* (Y402H) rs1061170:T>C Genotype**						
Genotype frequency	CC[Additive Model]	30 (24.7%)	11 (11%)	3.36	1.47-7.69	0.0036*
	TC[Additive Model]	57(47.1%)	47 (47%)	1.49	0.82-2.71	0.2273
	TT	34 (28.0%)	42 (42%)			
	CC vs TC+ TT[Recessive Model]			2.66	1.26-5.64	0.0093*
	CC+TC vs TT[Dominant Model]			1.85	1.05-3.24	0.0336*
Allele frequency	C	117 (48.34%)	69 (34.5%)	1.77	1.20-2.61	0.0037*
	T	125 (51.66%)	131 (65.5%)	0.56	0.38-0.82	0.0037*
LOC387715/ *ARMS2* (A69S) rs10490924: G>T Genotype						
Genotype frequency	TT[Additive Model]	40 (33.0%)	17 (17%)	3.20	1.45-7.07	0.0039*
	GT[Additive Model]	59 (48.7%)	53 (53%)	1.51	0.78-2.94	0.2426
	GG	22 (18.1%)	30 (30%)			
	TT vs GT+GG[Recessive Model]			2.41	1.26-4.59	0.0084*
	TT+GT vs GG[Dominant Model]			1.92	1.02-3.62	0.0553*
Allele Frequency	T	139 (57.44%)	87 (43.5%)	1.75	1.20-2.55	0.0041*
	G	103 (42.56%)	113 (56.5%)	0.57	0.39-0.83	0.0041*
***HTRA1* rs11200638:G>A Genotype**						
Genotype frequency	AA[Additive Model]	48 (39.6%)	25 (25%)	2.81	1.24-6.36	0.0147*
	GA[Additive Model]	58 (47.9%)	53 (53%)	1.60	0.75-3.41	0.2567
	GG	15 (12.3%)	22 (22%)			
	AA vs GA+GG [Recessive Model]			1.97	1.10-3.52	0.0223*
	AA+GA vs GG [Dominant Model]			1.99	0.97-4.08	0.0704
Allele Frequency	A	154 (63.64%)	103 (51.5%)	1.64	1.12-2.41	0.0118*
	G	88 (36.36%)	97 (48.5%)	0.60	0.41-0.88	0.0118*

OR: Odds Ratio; CI: Confidence Interval; *- Statistically significant susceptible genotype Additive Model: comparing mutant homozygous and heterozygous genotypes individually with wild homozygous genotypes; Recessive Model: comparing mutant homozygous genotype with wild homozygous and heterozygous genotype taken together; Dominant Model: mutant homozygous and heterozygous genotype taken together compared with wild homozygous genotype.

Table III: Linkage Disequilibrium association among *ARMS2G>T* and *HTRA1G>A* SNPs in AMD

	ARMS2G	*ARMS2T*	*HTRA1G*	*HTRA1A*
ARMS2G	*	0.9774	0.8227	0.6166
ARMS2T	0.9998	*	0.8381	0.8154
HTRA1G	0.885	0.9222	*	0.8482
HTRA1A	0.6756	0.8735	0.9998	*

Values below the diagonal represent standard LD values (D) generated using Lewontin's Principle; values above the diagonal represent correlation coefficient using Cramer's V statistic

DISCUSSION

We studied the association of variants in *CFH*, *ARMS2* and *HTRA1* genes for susceptibility of AMD in Indian population. The *CFH* Y402H gene frequencies of the risk C allele in Caucasians vary between 61%–94% in AMD cases and 34%–46% in controls[7-9]. In this study the C allele frequency in controls was 34.5% and CC genotype increased risk by 2.6 fold. An Indian study by Kaur *et al*.[24] has shown an odds ratio of 7.81 (95% CI 3.18 – 12.44) for CC genotype. The variation may be due to variations in populations as the study by Kaur *et al*. is from a centre in South India while the population in the present study was mainly from north India. The polymorphism Y402H in *CFH* (rs1061170) has been identified as a common non-synonymous variant and a major genetic risk factor for AMD development in other ethnic groups as well. According to Brantely *et al.*, the odds ratio for mutant homozygote (CC) is 6.5 and for heterozygote (TC) in Caucasians is 2.1[25]. In European population, Souied *et al.*, had found the odds ratio of 6.93 (95% CI 3.11- 15.46, p-value 0.0001). The allele frequency in these patients was 56.7% and in controls was 29% (p-value 0.0001)[26]. In a Chinese population study also the association was confirmed with AMD with an odds ratio of 3.23 (95% CI 1.56-6.70, *p*-value 0.005). However the mutant allele frequency in controls as well as patients was lower as compared to Caucasians[27]. Another study from northern China showed no association of *CFH* Y402H in AMD patients. The C allele was low in frequency (10.3% in cases and 8.0% in controls) and was not associated with AMD[28].

In this study, *HTRA1* and *ARMS2* gene polymorphisms also showed the association for the risk of AMD consistent with previous published findings from Caucasian populations[23]. Findings from this study showed that the AMD risk was higher with the T allele of *ARMS2* gene (rs10490924) and the A allele of *HTRA1* gene (rs11200638). This finding was supplemented by the LD results which revealed presence of mutant *ARMS2* T allele and HTRA1 A allele (D'=0.87, r=0.81) to

increase the risk of getting affected with AMD. Consistent with our findings, a small study from Poland has also reported odds ratio of 5.57 for ARMS2 T allele[29]. In the Indian study by Kaur *et al.,* the odds ratio of *HTRA1* and *ARMS2* genes with AMD were 2.70 and 2.92[23]. Similar ratios have been observed in our study. Odds ratio for *HTRA1* is 1.97, and for *ARMS2* (A69S) the odds ratio was 2.41. In the Chinese population according to Chu *et al.,* and Xu *et al.,* odds ratios for *HTRA1* gene were 3.98 and 7.90 respectively[27, 28]. Odds ratios as high as 15.5 have been reported by Leveziel *et al.,* in the French population[30]. In our study, odds ratio of *HTRA1* AA homozygotes is 1.97 with frequency of risk allele being 63.64% in AMD patients and 51.5% in controls, whereas European studies have reported higher odds ratios[31]. According to Xu *et al.,* in the northern Chinese population the odds ratio for *ARMS2* (A69S) gene was 5.45[28]. In the American population Ross *et al.,* reported an odds ratio of 8.59[31]. Presence of 4 or more mutant alleles was seen in few of the cases and controls in this study. Though the numbers are small, presence of 4 or more mutant alleles increased the odds ratio to about 7, indicating that each allele adds to the AMD risk independently. The data presented here confirms that the *CFH*, *ARMS2* and *HTRA1* genes are associated with susceptibility to AMD in the Indian population, as observed in other parts of the world. As AMD is a multifactorial disease, the interaction between these genetic variants and environmental factors may affect the development of different AMD phenotypes as well as its progression. The knowledge of the association of genetic factors with increased risk for AMD has substantially improved insight into the pathogenesis of this disease. In the future, determination of risk alleles may contribute to the identification of patients at risk and lead to the development of new therapeutic approaches.

In conclusion, this study suggests that all the three polymorphisms *CFH* Y402H, *ARMS2* A69S and the *HTRA1* G>A alleles are potential risk factors and they are independently associated with AMD in the Indian population. Detection of individuals at risk could lead to formulation of strategies for management and early prevention of this disease.

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ETHICAL STATEMENT

The study protocol was approved by the Institutional Ethics Committee (No. 1219/R-Cell-10).

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