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 years¹. 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 aggregations⁵. Twin and segregation analysis studies suggest a significant genetic contribution to the disease³⁴. Whole genome scans have led to identification of the association of AMD with chromosomal loci 1q, 9q, 10q, 16q and 22q by linkage analysis⁵⁷. Complement factor H (CFH) gene on chromosome 1q32 has been determined to be strongly associated with AMD⁷⁷. 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 H¹⁰. The Y402H polymorphism is in a region of CFH that binds heparin and C-reactive protein. CFH and its ligand C3b/C4b have been found to co-localize in amyloid-containing sub-structural elements within macular...
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.30.

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 (rs31200638) 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 Qanto 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 MgCl2, 2.5 µl, Taq 1 U/µl- 1.5µl, dNTP 10 milimoles - 1 µl, forward primer 10 picomoles-1µl, Reverse primer 10 picomoles - 1 µl, 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.1.25-27. PCR conditions were as follows: hot lid 105ºC for 5 min, denaturation 95ºC for 5 min, 35 cycles consisting of denaturation at 94ºC for 45 seconds, annealing for 45 seconds (at 50.7ºC for CFH, at 65.1ºC for ARMS2 and at 62.5ºC for HTRA1) and extension at 72 ºC for 1.5 minutes, followed by final extension at 72 ºC for 5 minutes and hold at 15ºC 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 NalIII (New England Biolabs, Ipswich, Massachusetts), for ARMS2 gene PCR product (158 bp) was digested with 2 units of Fnu4HI (New England Biolabs) and for HTRA1 gene PCR product 208 bp was digested with 2 units of MspI (New England Biolabs), incubated at 37ºC 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 (BioRad, 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.
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 \(< 0.05 \) 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 (Y402H), were included in this study.

<table>
<thead>
<tr>
<th>GENES / POLYMORPHISM</th>
<th>PRIMER SEQUENCE</th>
<th>PCR PRODUCT SIZE</th>
<th>ENZYME &amp; SIZES OF FRAGMENT AFTER DIGESTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFH-Y402H</td>
<td>5’TGTTCAGGTCACTTCTGCCTG3’</td>
<td>442 bp</td>
<td>Nla III Wild (T): 289+74+73+6bp</td>
</tr>
<tr>
<td>[rs1061170] T&gt;C</td>
<td>5’ACAAGGCTTACACATTTTGGC3’</td>
<td></td>
<td>Mutant (G): 203+86+74+73+6bp</td>
</tr>
<tr>
<td>HTRA1</td>
<td>5’CTCATGCCACCCACAAACAC-3’</td>
<td>208 bp</td>
<td>Msp I Wild (G): 141+44+23bp</td>
</tr>
<tr>
<td>[rs11200638] G&gt;A</td>
<td>5’GTCATTGCCATGCTTCG-3’</td>
<td></td>
<td>Mutant (A): 185+23bp</td>
</tr>
<tr>
<td>ARMS2</td>
<td>5’CTGCAAGGAGGACCGACTGAC3’</td>
<td>158 bp</td>
<td>Fnu4 HI Wild (G): 64+71+23bp</td>
</tr>
<tr>
<td>Or LOC387715 [rs10490924] G&gt;T</td>
<td>5’CTGCTTGCAGGTGCTGGA3’</td>
<td></td>
<td>Mutant (T): 135+23bp</td>
</tr>
</tbody>
</table>

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.
The frequency in these patients was 56.7% and in 23 types individually with wild homozygous genotypes; Recessive Model: comparing mutant homozygous and heterozygous genotype taken together compared with homozygous genotype with wild homozygous and heterozygous genotype taken together; Dominant Model: mutant homozygous and heterozygous genotype compared with wild homozygous genotype.

According to Brantely et al., the odds ratio for mutant homozygote (CC) is 6.5 and for heterozygote (TC) in Caucasians is 2.1.75 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). The allele frequency in patients was lower as compared to controls as well as patients was lower as compared to Caucasians.75. 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 4% in controls) and for heterozygote (TC) in Caucasians is 2.1. In 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.77. 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.86.

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.93. 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

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

<table>
<thead>
<tr>
<th>Genotype frequency</th>
<th>AMD patients (N=121)</th>
<th>Controls (N=100)</th>
<th>OR</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC (Additive Model)</td>
<td>30 (24.7%)</td>
<td>11 (11%)</td>
<td>3.36</td>
<td>1.47-7.69</td>
<td>0.0036*</td>
</tr>
<tr>
<td>TC (Additive Model)</td>
<td>57 (47.1%)</td>
<td>47 (47%)</td>
<td>1.49</td>
<td>0.82-2.71</td>
<td>0.2273</td>
</tr>
<tr>
<td>TT</td>
<td>34 (28.2%)</td>
<td>42 (42%)</td>
<td>2.66</td>
<td>1.26-5.64</td>
<td>0.0093*</td>
</tr>
<tr>
<td>CC vs TT (Recessive Model)</td>
<td>117 (48.34%)</td>
<td>69 (69.5%)</td>
<td>1.85</td>
<td>1.05-3.24</td>
<td>0.0336*</td>
</tr>
<tr>
<td>CC+TC vs TT (Dominant Model)</td>
<td>117 (48.34%)</td>
<td>69 (69.5%)</td>
<td>1.77</td>
<td>1.20-2.61</td>
<td>0.0037*</td>
</tr>
<tr>
<td>Allele frequency</td>
<td>C</td>
<td>125 (51.66%)</td>
<td>131 (65.5%)</td>
<td>0.56</td>
<td>0.38-0.82</td>
</tr>
<tr>
<td>TT</td>
<td>125 (51.66%)</td>
<td>131 (65.5%)</td>
<td>0.56</td>
<td>0.38-0.82</td>
<td>0.0029*</td>
</tr>
</tbody>
</table>

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

| 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 |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| ARMSSG | 0.0774 | 0.8227 | 0.6166 |
| ARMSST | 0.9998 | * | 0.8381 | 0.8154 |
| HTRAG | 0.885 | 0.9222 | * | 0.8482 |
| HTRA1A | 0.6756 | 0.8735 | 0.9998 | * |

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.99. 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 2.1.75. 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). The allele frequency in patients was lower as compared to Caucasians.75. 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.86.

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.93. 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

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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\(^2\). In the Indian study by Kaur et al., the odds ratio of HTRA1 and ARMS2 genes with AMD were 2.70 and 2.92\(^3\). 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\(^7\)-\(^8\). Odds ratios as high as 15.5 have been reported by Leveziel et al., in the French population\(^9\). 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\(^10\). According to Xu et al., in the northern Chinese population the odds ratio for ARMS2 (A69S) gene was 5.45\(^11\). In the American population Ross et al., reported an odds ratio of 8.59\(^12\). 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.

**Acknowledgements**

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**Ethical Statement**

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

**References**


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3752