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# Vascular endothelial growth factor gene polymorphisms and association with age related macular degeneration in Indian patients

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ABSTRACT

*Background:* Age-related macular degeneration (AMD) is an important cause of visual impairment in elderly people. AMD is a multifactorial 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:* This study was aimed to investigate the association of polymorphisms in *VEGF* genes with age related macular degeneration (AMD) in Indian patients.
*Method:* Genotyping for the *VEGF* −1154 (G > A), −2578 (C > A), +405 (G > C) and −460 (C > T) SNPs was performed in 100 AMD patients and 100 controls by polymerase chain reaction (PCR), restriction fragment length polymorphism (PCR-RFLP) and sequencing method.
*Results:* Out of the four SNPs, heterozygous genotypes of VEGF −1154 G > A (OR = 2.58, $p = 0.0035$), +460 C > T (OR = 2.90, $p = 0.0046$), and +405 G > C (OR = 2.02, $p = 0.02$) have shown susceptible association with AMD. However, *VEGF* −2578 C > A did not show any statistical significance. Further A-A-G-T haplotype comprising of three mutant alleles revealed risk association (OR = 12.7, $p = 0.0030$) with AMD.
*Conclusion:* The present study suggests significant genetic associations for VEGF −1154 G > A, +460 C > T, and +405 G > C polymorphisms with AMD. Early detection of individuals with risk to these SNPs could lead to strategies for prevention, early diagnosis, and management of AMD.



## 1. Introduction

Age-related macular degeneration (AMD; MIM, 603075) is a multifactorial neurodegenerative disease of retina that causes progressive impairment of central vision and is a leading cause of irreversible vision loss in elderly people. Though both environment and genetic factors are implicated; its actual etiology is largely unknown. Polymorphisms in *CFH*, *ARMS2* and *HTRA1* have been consistently shown to be associated with AMD across different populations. In addition, other genes like *ApoE, CX3CR1, PLEKHA1* and *VEGF* were studied for their association with AMD. Neovascularization is an important component of pathology of AMD. Vascular endothelial growth factor (*VEGF-A*), is a major player in the control of angiogenesis. *VEGF* is a secreted endothelial specific mitogen, which acts as a key regulator of vascular permeability. *VEGF* 165 is the most predominant isoform reported in eye (Perrin et al., 2005). *VEGF* 165a has potent angiogenic properties. It has also been found to play a key role in choroidal neovascularization (Witmer et al., 2003). Current treatments that inhibit *VEGF* activity are effective in improving vision in ~40% of affected neovascular AMD cases (Oishi et al., 2011). High expression of *VEGF* has also been recognized as an important factor promoting neovascularization in wet AMD (Frank et al., 1996). Several single nucleotide polymorphisms (SNPs) in the *VEGF* gene affect its expression. Association of *VEGF* with AMD has been looked for in some populations. Common *VEGF* polymorphisms like g.43737830 A > G (*VEGF* −1154G > A), g.3437 A > C (*VEGF* −2578C > A), and g.43737486C > T (*VEGF* −460C > T) are found in the promotor region and g.43710613 (VEGF −634G > C or VEGF +405G > C) is found in the 5'UTR region, of the VEGF gene (Awata et al., 2002). The frequency of these polymorphisms varies across different populations and associations with AMD have been reported in some studies (Lin et al., 2008; Haines et al., 2006; Churchill et al., 2006). Keeping these associations in mind, the current study was designed to investigate the association

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of these single nucleotide polymorphisms (SNPs) and their haplotypes with AMD patients from India.

## 2. Materials and methods

### 2.1. Patients and clinical examination

The study protocol was approved by the Ethics Committee of Sanjay Gandhi Post Graduate Institute of Medical Sciences, Raibareli road, Lucknow, Uttar Pradesh India. 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 AMD were included. Controls were >70 years of age and were confirmed not to have clinical evidence of AMD in any of the eyes by opthalmological examination. Sample size was calculated before the study with the use of Quanto software version 1.2 by institutional statistician for the 80% power of the study; the sample size was calculated to be 92. Samples were collected after obtaining informed consent from 100 clinically diagnosed AMD cases and 100 controls. There remained no conflict of interest pertaining to this study.

### 2.2. DNA extraction and genotyping

Five milliliter peripheral venous EDTA blood was collected from all participants and stored at −80 °C. 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, restriction fragment length polymorphism (PCR-RFLP) and sequence based typing (SBT) methods. The reaction mixture common for all four genotypes consisted of: 10× buffer with $MgCl_2$ 2.5 μl, Taq 1 U/μl - 1.5 μl, dNTP 10 milimoles - 1 μl, forward primer 10 picomoles (pmol) - 1 μl, reverse primer 10 pmol - 1 μl, DNA – 1.5 μl (50 ng/μl) and HPLC water 16.5 μl per 25 μl of the reaction mixture. Details of the primers and enzymes used in the study for each *VEGF* SNPs are given in Table 1. 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 s, annealing for 45 s (65.5 °C for −2578C > A, at 62.0 °C for −1154G > A, at 50.7 °C for +405G > C and at 63.0 °C for −460C > T) and extension at 72 °C for 1.5 min, followed by final extension at 72 °C for 5 min and final hold at 15 °C. The amplified products were tested on 2% agarose gel pre-stained with ethidium bromide (EtBr) with a migrating distance of approximately 3 cm. Samples were then used for RFLP and SBT.

Three polymorphisms namely, −1154G > A, −2578C > A, and +405G > C VEGF SNPs were studied by PCR-RFLP method using restriction enzymes *Mnl*I, *Bgl*II and *BsmF*I (10 U) (New England Biolabs) respectively. PCR products were incubated with the respective enzymes at 37 °C overnight. The digested products were run on 15% polyacrylamide gel electrophoresis (PAGE), stained with EtBr. Gel images were taken using the molecular imager gel doc XR System (Bio-Rad, Hercules, CA). Genotypes were determined based on their restriction patterns. In case of −1154G > A, three fragments (150 bp, 34 bp, 22 bp) were observed for wild G allele and two fragments (184 bp, 22 bp) for the variant A allele (Fig. 1(A)). The mutant allele A of *VEGF* −2578C > A SNP was digested into 202 bp and 122 bp fragments while the wild allele C remained uncut with a length of 324 bp (Fig. 1(B)). The wild type G allele of *VEGF* +405G > C remained uncut, with a length of 431 bp, whereas its mutant allele C was fragmented into 153 bp and 278 bp (Fig. 1(C)). *VEGF* −460C > T (336 bp) genotyping was done by SBT (Supplementary Fig. 1) using ABI 310 sequence analyzer (Applied Biosystems Corporation, CA, USA). 10% of the studied samples were re-validated by genotyping using double blind method (Supplementary Figs. 2, 3, & 4).

### 2.3. Statistical analysis

The differences in the frequency of *VEGF* genotypes, alleles and haplotypes between the study and control groups were calculated using Fisher exact test. *P* values ≤ 0.05 were considered statistically significant. Statistical analysis was done using commercial statistical analysis software (GraphPad Software ver. 3.05; GraphPad, San Diego, USA) to calculate the odds ratio (OR) in order to measure the strength of associations between genotypes, allele frequencies and haplotypes. Haplotypes and pair-wise linkage disequilibrium were generated using Arlequin software (v 3.5) (University of Dusseldorf, Germany) (Excoffier and Lischer, 2010). A negative Tajima's D signifies an excess of low frequency polymorphisms relative to expectation, indicating population size expansion or purifying selection. A positive Tajima's D signifies low levels of low and high frequency polymorphisms, indicating a decrease in population size or balancing selection (Tajima, 1989). Allele frequencies were calculated as the number of occurrences of the test allele in the population divided by the total number of alleles. F-SNP approach was utilized to evaluate the functional relevance of the studied *VEGF* SNPs *in-silico* (Imai et al., 2010).

## 3. Results

### 3.1. Genotype and allele frequency

The genotype and allele frequencies were found to be in Hardy Weinberg equilibrium in normal healthy controls. Functional evaluation of SNPs studied from F-SNP showed that all the four SNPs are having effect on the regulation of transcription. Genotype frequencies were compared by applying the additive, dominant and recessive models (Table 2). The occurrence of heterozygous genotype (GA) of the −1154G > A polymorphism was significantly higher in AMD cases compared to that of controls (OR = 2.58, 95% CI = 1.39–4.78, $P = 0.0035$). The dominant model of inheritance showed risk association for AMD cases (OR = 2.26, 95% CI = 1.24–4.13, $P = 0.0112$) compared to that of the controls. The frequency of variant (AA) genotype of −2578C/A polymorphism was higher in the AMD cases as compared to controls. However, no statistical significance was observed for the AA genotype. None of the models of inheritance showed statistical significance for −2578C > A. For the +405G > C the heterozygous genotype GC showed susceptible association (OR = 2.02, 95% CI = 1.13–3.61, $P = 0.0200$). But the difference between allele frequencies was not statistically

**Table 1**
Primer sequences and restriction enzymes used in the study.

| S.no | Polymorphism | Primer sequence (5′ → 3′) | PCR product size | Enzyme | After digestion |
|---|---|---|---|---|---|
| 1. | *VEGF*-1154G > A (rs1570360) | 5′-TCCTGCTCCCTCCTCGCCAATG-3′<br>5′-GGCGGGGACAGGCGAGCATC-3′ | 206 bp | *Mnl* I | Wild (G)- 150 + 34 + 22 bp<br>Mutant (A)- 184 + 22 bp |
| 2. | *VEGF* −2578C > A (rs699947) | 5′-GGATGGGGCTGACTAGGTAAGC-3′<br>5′-AGCCCCCTTTTCCTCCAAC-3′ | 324 bp | *Bgl* II | Wild (C)- 324 bp<br>Mutant (A)- 202 + 122 bp |
| 3. | *VEGF* + 405G > C (rs2010963) | 5′- GATTGCTCTACTTCCCCAAATC −3′<br>5′- CTCCTCCGAAGCGAGAACAG −3′ | 431 bp | *BsmF* I | Wild (G)- 431 bp<br>Mutant (C)- 153 + 278 bp |
| 4. | *VEGF* −460C > T (rs833061) | 5′- GCCCATTCCCTCTTTAGCCA-3′<br>5′- GGGAGCAGGAAAGTGAGGTT-3′ | 336 bp | Sequencing | – |