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

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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.

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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 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 −1154 G>A), g.3437 A>C (VEGF −2578 C>A), and g.43737486C>T (VEGF −460 C>T) are found in the promoter region and g.43710613 (VEGF −634 G>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...
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 ophthalmological 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 μl buffer with MgCl2 2.5 μl, Taq 1 U/μl, dNTP 100 μmol - 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 SNP 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 −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 migration 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 MnlI, BgII and BsmFI (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 (Inai 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 relative 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

| S.no | Polymorphism | Primer sequence (5′ → 3′) | PCR product size (bp) | Enzyme | After digestion |
|------|--------------|--------------------------|-----------------------|--------|----------------|
| 1.   | VEGF−1154G > A | 5′-TCCTGTCTCCTCTGCGCCATG-3′ | 206 bp | MnlI | Wild (C)– 150 + 34 + 22 bp |
|      | (rs1570360)   | 5′-GGCCGGCACAGGCGAGCTAC-3′ | Mutant (A) – 184 + 22 bp |
| 2.   | VEGF −2578C > A | 5′-GCTATGGGCTGCTAGTTATACG-3′ | 324 bp | BglII | Wild (C) – 324 bp |
|      | (rs699947)    | 5′-AGGCCCCCTTCTCCCAAAC-3′ | Mutant (A) – 202 + 122 bp |
| 3.   | VEGF +405G > C | 5′-GATGCTCTAATTCTTCTTATC-3′ | 431 bp | BsmFI | Wild (G) – 431 bp |
|      | (rs2010963)   | 5′-CTCTCCAGGAGGCGACACGG-3′ | Mutant (C) – 153 + 278 bp |
| 4.   | VEGF −460C > T | 5′-GGCCATTTCCCTGCTTTGCA-3′ | 336 bp | Sequencing | |
significant. Mutant TT genotype and heterozygous CT genotype at −460C>T showed significant association with odds ratios of 3.26 and 2.9 respectively. Significant risk association was also concurred for AMD cases under the dominant model of inheritance (OR = 3.00, 95% CI = 1.11–2.45, P = 0.0163) and C allele (OR = 0.60, 95% CI = 0.40–0.89, P = 0.0163) of −460C>T SNP. None of the alleles at other three SNPs revealed level of significance for AMD in our study group.

3.2. Haplotype frequency

The haplotype analysis revealed the presence of sixteen and fourteen haplotypes in the cases and controls respectively. Of these twelve haplotypes were commonly found among both patient and control groups. The haplotypes that were dominantly observed and the ones which had protective or risks predisposing effect have been shown in Table 2. Intriguingly only one haplotype, A-A-G-T which has mutant alleles at 3 of 4 loci and wild allele at +405 showed significant risk association (OR = 12.7, P = 0.0030). While, C-G-C-C haplotype (OR = 0.10, P = 0.0200) which has wild alleles at 3 of 4 loci and mutant allele at +405 revealed protective association among AMD cases.

3.3. Linkage disequilibrium

Pair wise linkage disequilibrium (LD) analysis was carried out to estimate the Tajima’s LD measure (D) and correlation coefficient using Cramer’s V statistic (r). The LD analysis revealed Tajima’s D values > 0 among the studied mutant VEGF alleles suggesting plausible role of multiple alleles in AMD progression and causation (Table 4). The pair wise analysis revealed VEGF −2578 A −1154 A (D = 0.1852, r = 0.7709), VEGF −2578 A + 405C (D = 0.1937, r = 0.8036), VEGF −2578 A −460 T (D = 0.1960, r = 0.8449), VEGF −1154 A + 405C (D = 0.1991, r = 0.8072), VEGF −1154 A −460 T (D = 0.1824, r = 0.8986).

Table 2: Genotype and allele frequencies for VEGF −1154 G > A, −2578C > A, −405G > C and −460C > T polymorphisms among AMD patients vs controls.

| Genotype | AMD patients (N = 100) | Controls (N = 100) | OR       | 95% CI       | p-Value |
|----------|------------------------|-------------------|----------|-------------|---------|
| VEGF − 1154 G > A Genotype | | | | | |
| Genotype frequency | 0.93 | 0.30–2.84 | 1.0000 |
| GA [additive model] | 11 (11%) | 0.93 | 0.30–2.84 | 1.0000 |
| GG | 46 (46%) | 2.58 | 1.39–4.78 | 0.0039* |
| AA vs GA GCC [recessive model] | 0.51 | 0.18–1.45 | 0.3105 |
| AA + GA vs GC [dominant model] | 2.26 | 1.24–4.13 | 0.0112* |
| Allele frequency | 1.32 | 0.87–1.98 | 0.2145 |
| A | 119 (59.5%) | 0.75 | 0.50–1.13 | 0.2145 |
| G | 68 (34%) | 1.32 | 0.87–1.98 | 0.2145 |
| VEGF − 2578 A > C Genotype | | | | | |
| Genotype frequency | 2.03 | 0.86–4.79 | 0.1326 |
| CA [additive model] | 15 (15%) | 2.03 | 0.86–4.79 | 0.1326 |
| CC | 53 (53%) | 1.56 | 0.80–3.02 | 0.1890 |
| AA vs CA GCC [recessive model] | 1.50 | 0.72–3.12 | 0.3576 |
| AA + CA vs GC [dominant model] | 1.66 | 0.88–3.14 | 0.1514 |
| Allele frequency | 1.38 | 0.93–2.05 | 0.1319 |
| A | 117 (58.5%) | 0.72 | 0.48–1.07 | 0.1319 |
| C | 83 (41.5%) | 1.38 | 0.93–2.05 | 0.1319 |
| VEGF + 405 A > G Genotype | | | | | |
| Genotype frequency | 0.29 | 0.06–1.43 | 0.1925 |
| GC [additive model] | 9 (9%) | 0.29 | 0.06–1.43 | 0.1925 |
| GG | 53 (53%) | 2.02 | 1.13–3.61 | 0.0020* |
| CC vs GC GCC [recessive model] | 0.20 | 0.04–0.98 | 0.0582 |
| CC + GC vs GG [dominant model] | 1.69 | 0.96–2.96 | 0.0886 |
| Allele frequency | 1.15 | 0.75–1.77 | 0.5837 |
| C | 144 (72%) | 0.86 | 0.56–1.33 | 0.5837 |
| G | 56 (28%) | 1.15 | 0.75–1.77 | 0.5837 |
| VEGF − 460 T > C Genotype | | | | | |
| Genotype frequency | 3.26 | 1.35–7.86 | 0.0103* |
| TT [additive model] | 19 (19%) | 3.26 | 1.35–7.86 | 0.0103* |
| CT [additive model] | 50 (50%) | 2.90 | 1.37–6.14 | 0.0046* |
| CC | 31 (31%) | 1.49 | 0.76–2.92 | 0.3096 |
| TT vs CT + CC [recessive model] | 3.00 | 1.46–6.18 | 0.0034* |
| TT + CT vs CC [dominant model] | 1.65 | 1.11–2.45 | 0.0163* |
| Allele frequency | 0.60 | 0.40–0.89 | 0.0163* |
| G | 112 (56%) | 1.65 | 1.11–2.45 | 0.0163* |
| T | 88 (44%) | 0.60 | 0.40–0.89 | 0.0163* |

OR: Odds Ratio; CI: Confidence Interval; * - Statistically significant protective genotype; # - 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 genotypes taken together and compared with wild homozygous genotype.
Values below the diagonal represent standard LD values (D) generated using Tajima’s Principle; values above the diagonal represent correlation coefficients.

Table 3
Haplotype distribution among AMD cases and normal healthy controls.

| Haplotype | Patient (n = 100) | Control (n = 100) | OR (95% CI) | p-Value |
|-----------|-------------------|------------------|-------------|---------|
| CGCT      | 52                | 39               | 1.45        | 0.90-2.32 | 0.1521 |
| CAGC      | 55                | 50               | 1.13        | 0.72-1.77 | 0.6496 |
| CAGC      | 4                 | 6                | 0.655       | 0.18-2.37 | 0.7508 |
| CGGT      | 32                | 39               | 0.7863      | 0.46-1.31 | 0.4325 |
| CAGC      | 1                 | 9                | 0.10        | 0.01-0.85 | 0.0209* |
| AACT      | 2                 | 2                | 1.00        | 0.13-7.17 | 1.3769 |
| AGGC      | 17                | 25               | 0.65        | 0.33-1.24 | 0.2534 |
| CACT      | 1                 | 1                | 1.00        | 0.062-16.11 | 1.5013 |
| AAGT      | 12                | 1                | 12.70       | 1.83-98.69 | 0.0030* |
| AAGC      | 6                 | 3                | 2.031       | 0.50-8.23 | 0.5028 |
| AACC      | 7                 | 1                | 0.497       | 0.04-5.53 | 1.0000 |
| AGGT      | 7                 | 3                | 2.38        | 0.60-9.34 | 0.3175 |

OR: Odds Ratio; CI: Confidence Interval; *- Statistically significant protective genotype; #- Statistically significant susceptible genotype.

0.7683) and VEGF + 405C − 460 T (D = 0.1632, r = 0.6854) alleles to be in positive LD and justified by the significant “r values”.

3.4. F SNP results

All the studied VEGF SNPs were found to have minor or major role in the transcription regulation process. FS Score 0.176, 0.242, 0.257 and 0.282, were observed against −2578C > A, −1154G > A, +405G > A and −460C > T SNPs. The findings suggest a significant change in the transcription regulation due to frame shift coding (FS score = 0.28) in −460C > T SNP. Further in-silico analysis revealed changes in transcription regulation pattern for −1154G > A (FS score = 0.24) (Table 3).

4. Discussion

Understanding about etiology of AMD is yet limited. Genetic factors controlling angiogenesis and inflammation are being investigated for their role in pathogenesis of AMD. The angiogenesis process is highly controlled through the balance of pro- and anti-angiogenic factors. VEGF being an important player in the growth of new blood vessels is the key pro-factor in this process and probably a factor in development and progression of AMD. The new blood vessels tend to leak fluid and blood under the macula, leading to the loss of central vision. A number of therapies have been developed to block the effects of VEGF by binding to and sequestering the protein. Hence VEGF polymorphisms are likely to be causally associated with AMD. Previous studies investigated the possible associations between VEGF-A gene polymorphisms and AMD, with contrasting data. Out of four polymorphisms three have shown association with AMD in this study with odds ratios varying from 2.02 to 3.26. The −2578C > A has not been associated with AMD in our study as was the case in Japanese and Caucasians (Amy et al., 2009; Lee and Shatky, 2009).

In the current study association of VEGF − 1154G > A and −460C > T SNPs were observed with AMD. Odds ratios for mutant homozygotes and heterozygote under dominant mode for −460C > T SNP was 3, while that for VEGF − 1154G > A was 2.26. Studies in Japanese population did not show association of VEGF − 1154G > A polymorphism (Awata et al., 2002). Haines et al. have showed risk association for variant genotypes of −460C > T SNP with AMD (Haines et al., 2006) which is in lieu with our findings. Studies by Janik-Papis et al. (2009) and Szaflik et al. (2009) in the Polish population have also showed similar risk association for AMD with heterozygous −460 CT genotype. A strong association of A−G−T haplotype (odds ratio of 12) with AMD suggests the possibility of additive independent association of VEGF − 1154 G > A and 460C > T alleles shown in Table 3.

VEGF + 405G > C is a well studied polymorphism in AMD but still there is no consensus. Studies from Tunisia and Poland showed association of VEGF + 405G > C (Janik-Papis et al., 2009; lmen et al., 2014). In contrast, one study on Taiwan Chinese population has showed insignificant association of the +405 C allele with AMD (Lin et al., 2008). Churchill et al. also did not find the independent association with VEGF + 405 SNP but the +405C allele was present in the haplotype association with neovascular AMD (Churchill et al., 2006). In the present study, heterozygote +405 GC genotype showed odds ratio 2.02 (P value of 0.02). Pathology of AMD and diabetic retinopathy has similarities in the form of neovascularity. Hence, increased VEGF level may be seen as a pathogenetic factor is likely to be a commonality in the pathogenesis. A study in diabetic retinopathy by Awata et al. showed association of +405 C genotype with diabetic retinopathy (Awata et al., 2002). They also showed that healthy individuals with +405 CC genotype have higher levels of VEGF. A study on Tunisian population has showed the plasma levels of VEGF + 405 CC genotype were statistically higher in AMD patients than in controls. However, another study showed that the healthy subjects with GG genotype showed highest production of VEGF after stimulation of mononuclear cells with lipopolysaccharide (Watson et al., 2000). This indicates that the effect of alleles at +405 on VEGF level is not consistent or not significant. There is one study from India and showed the association in CG heterozygous genotype in Indian population (Suganthalakshmi et al., 2006). This is similar to our results. The inconsistent results from studies from different countries may be due to differences in the sizes of the studies and the population based variations.

VEGF is a proven growth factor and prominent cytokine that plays a role in pathogenesis of AMD. Efficacy of anti VEGF antibody in the treatment of AMD supports this hypothesis. The present study and some other studies signifies genetic associations of VEGF − 1154G > A, +405G > C and −460C > T SNPs with AMD. However, the effects of the various SNPs in VEGF gene, singly or in combinations on the VEGF level production need to be studied. We have not studied VEGF levels; which remains as a major limitation of the study.

Financial disclosures

None.

Declaration of interest

There are no conflicts of interest.
Table 5
Functional evaluation of VEGF.

| Genetic variation | Chromosomal Pos | Functional category | Prediction tool | Prediction result | FS score |
|-------------------|-----------------|---------------------|----------------|------------------|---------|
| VEGF – 1154G > A  | Chr 6, 43,845,808 | Transcription regulation | TFSearch       | Change           | 0.242   |
| rs1570360         |                 |                     |                |                  |         |
| VEGF – 2578C > A  | Chr 6, 43,844,367 | Transcription regulation | TFSearch       | Change           | 0.176   |
| rs699947          |                 |                     |                |                  |         |
| VEGF – 405G > C   | Chr 6, 43,846,328 | Transcription regulation | TFSearch       | Change           | 0.257   |
| rs2010963         |                 |                     |                |                  |         |
| VEGF – 460C > T   | Chr 6, 43,845,464 | Transcription regulation | TFSearch       | Frame shift coding | 0.282 |
| rs833061          |                 |                     |                |                  |         |

Contribution of authors

The work was designed by Shubha Phadke, Vani Gupta, Vinita Singh and Shobhit Chawla. Research work was performed by Divya Gupta and Swayam Prakash. Diagnosis of AMD patients and control samples was done by Shobhit Chawla. The paper was edited by Shubha Phadke, Vani Gupta, Shobhit Chawla and Suraksha Agrawal.

Statement for the submission

We are submitting an original manuscript in your esteemed journal “Journal of Meta Gene”. This work has not been published anywhere previously and that it is not simultaneously being considered for any other publication.

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Appendix A. Supplementary data

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