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Association of *VEGF* and *eNOS* gene polymorphisms in type 2 diabetic retinopathy

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Purpose: Vascular endothelial growth factor (VEGF) is a major mediator of angiogenesis, and nitric oxide (NO) is an upstream and downstream regulator of VEGF mediated angiogenesis. VEGF and NO have been suggested to play an important role in the pathogenesis of microvascular complications in diabetic retinopathy (DR). The objective of this study was to examine the genetic variations of the *VEGF* and *eNOS* gene and assess their possible relationship to DR in type 2 diabetic patients in the Indian population.

Methods: In this study, 210 unrelated patients were enrolled and categorized into two study groups: a DR group, consisting of patients with proliferative diabetic retinopathy, and a diabetic without retinopathy (DWR) group comprised of patients with type 2 diabetes of more than 15 years duration who showed no signs of DR or had fewer than five dots or blot hemorrhages. Association of the genetic polymorphisms in the promoter and 5' UTR region of *VEGF* and the intron4 region of *eNOS* were studied. Total genomic DNA was isolated from peripheral blood leukocytes. PCR-RFLP analysis was performed for all samples to evaluate the genotypes. The distributions of the genotypes were compared using the χ^2 test. Haplotype estimation and multiple logistic regression analysis were carried out to analyze the significance of polymorphisms.

Results: We investigated four reported polymorphisms in the *VEGF* (5' UTR, promoter) and one reported polymorphism (intron 4) in the *eNOS* gene in Type 2 diabetes patients with (n=120) and without (n=90) retinopathy. The genotype distribution of the C(-7)T, T(-1498)C, and C(-634)G polymorphisms of *VEGF* differed significantly between patients with DR and DWR (p=0.001, p=0.0001, and p=0.021, respectively). Allele C in the -1498 region (p=0.0001) and T in -7 region (p=0.002) were also found to be significantly increased in patients with retinopathy. Calculated odds ratios (OR) for three heterozygous genotypes of C(-7)T, T(-1498)C, and C(-634)G regions were 4.17 (95% CI: 1.90-9.18, p=0.0001), 4.37 (95% CI: 2.44-7.84, p=0.0001), and 2.33 (95% CI: 1.24-4.36, p=0.008), respectively, and was found to be significantly higher in the DR group when compared with the DWR group. Multiple logistic regression analysis revealed that the nongenetic parameters, age (p=0.024) and duration of diabetes (p=0.009), and the genetic parameters, like *VEGF* C(-7)T (p=0.002) and T(-1498)C (p=0.001) polymorphisms, were significantly associated with DR. The frequencies of haplotype consisting of the majority of alleles in *VEGF* were found to be significantly associated with DR. The genotype distribution of *eNOS* did not differ significantly between the two study groups, and therefore the *eNOS* intron 4 polymorphism was considered to be less significant.

Conclusions: This is the first study to report *VEGF* and *eNOS* gene polymorphisms in patients with DR in the Indian population. The data suggest that the polymorphisms in the 5' UTR and promoter region of *VEGF* could be regarded as a major genetic risk factor for DR.

Diabetic retinopathy (DR), a devastating microvascular complication in the eye, is one of the leading causes of blindness in the western world but its frequency varies in different ethnic groups. As the prevalence of diabetes is high among asian Indians, studies on diabetic complications are of considerable clinical significance [1,2].

Most diabetic patients, especially those with poor glycemic control, develop DR, which remains the major cause of onset of blindness among diabetic adults [3]. The Wisconsin Epidemiologic Study of Diabetic retinopathy (WESDR) showed that 28.8% of diabetic patients develop retinopathy early, whereas 22.2% with the history of diabetes irrespective of glycemic exposure do not develop retinopathy. This study suggested that genetic factors could promote the onset of retinopathy in diabetic patients [4]. DR is characterized by vascular permeability, increased tissue ischemia, and angiogenesis. VEGF is a 45 KDa homodimeric glycoprotein [5], which has been shown to be an important mediator of retinal ischemia-associated intraocular neovascularization [6]. Many cell types within the eye produce vascular endothelial growth factor (VEGF). In patients with proliferative diabetic retinopathy (PDR), VEGF levels are markedly elevated in the vitreous and aqueous fluids [7,8]. Retinal VEGF expression is high in DR [9,10] and appears to be an attractive candidate gene for DR. The human *VEGF* gene is organized into eight exons sepa-

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rated by seven introns and is located on chromosome 6 [11].

Another possible factor for diabetes is nitric oxide (NO). The gene encoding *eNOS* (endothelial nitric oxide synthase) is located on chromosome 7q35-36. NO causes increased oxidative stress due to free radicals and could play an important role in the pathogenesis of microvascular complications in humans [12,13]. NO production has been reported to be either increased or decreased in the presence of high glucose concentrations [14,15]. NO is a short-lived, highly reactive intercellular signaling molecule. In addition to its antithrombogenic and antiplatelet regulatory activities, NO plays a major role in the regulation of vascular tone, including retinal circulation and vascular remodeling [16,17]. The angiogenic and inflammatory effects of VEGF can be mediated by NO, which is produced by VEGF-activated eNOS in vascular endothelial cells. This suggests that eNOS is involved in inflammation, ischemic processes, and the pathogenesis of DR [18-20]. Evidence suggests that *VEGF* and *eNOS* are associated with DR in different ethnic groups. To our knowledge, no information about this association is available in the Indian population. Hence we investigated the *VEGF* and *eNOS* to assess their possible relationships to DR in type 2 diabetic patients in the Indian population.

METHODS

Clinical subjects: This study was conducted over 2003-2004 with the approval of our Institutional Review Board and in accordance with the guidelines of the Declaration of Helsinki. Informed consent was given by all patients. All patients had type 2 diabetes, as defined by age of onset being later than 30 years of age, and who controlled their diabetes exclusively with oral hypoglycemic agents. All were subjected to a clinical evaluation which involved fundus examination by a binocular indirect ophthalmoscope and a slit lamp biomicroscopic examination with a 90 D lens in the retina clinic. Patients with either no signs of DR or with fewer than five dots or blot hemorrhages coupled with type 2 diabetes of >15 years duration were assigned to the diabetic without retinopathy (DWR) study group. Patients with PDR and more severe hemorrhages were allocated to the DR study group. Other patient details such as age, sex, body weight, duration of diabetes, age at onset of diabetes, family history of diabetes, other systemic illness, and

Table 1. Patient parameters for this study

Clinical characteristics	Diabetic retinopathy	Diabetic without retinopathy	p
Number of patients	120	90	
Males (%)	56.6	77.7	0.001
Females (%)	43.4	22.3	
Mean age (years)	63 ± 7.2	59 ± 8.7	0.0003
Mean age at onset (years)	39.5 ± 8.0	41 ± 6.3	0.1432
Mean duration of diabetes (years)	15.5 ± 6.9	17 ± 8.0	0.1470

The different parameters were compared between patients with and without retinopathy. Ages and durations are shown ±standard deviation.

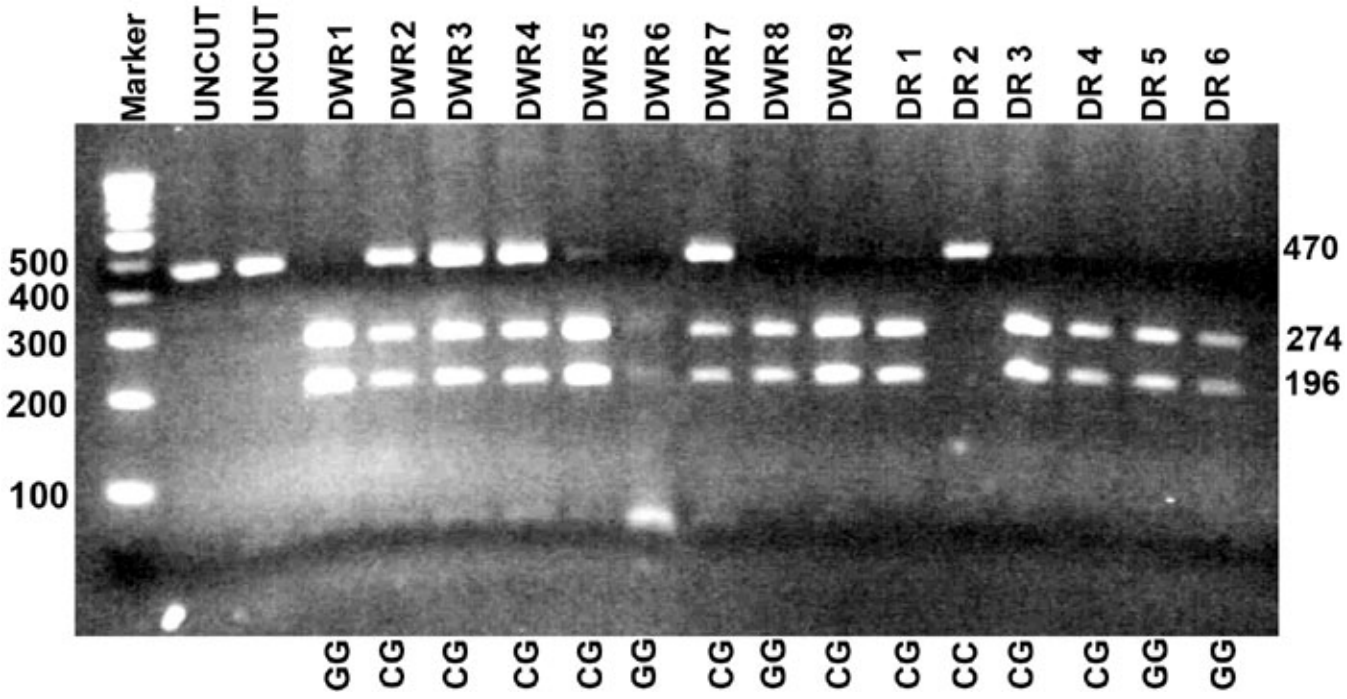

Figure 1. RFLP analysis for *VEGF* C(-634)G polymorphism. *BsmF1* restriction enzyme analysis showing the genotypes of the *VEGF* C(-634)G region, separated on a 3% agarose gel. Fragment size for each allele given in Table 2. The 470 bp band indicates the C allele, the 274 and 196 bp band indicates the G allele, and the 470 bp, 274 bp, and 196 bp bands indicate the CG genotype. Based on this banding pattern the genotype is provided below for each well. The molecular marker is a 100 bp ladder.

treatment details were also documented. All the patients in addition underwent 5-field 50° color fundus photography.

DNA preparation: Total genomic DNA was extracted from peripheral blood leucocytes by the salt precipitation method [21] from all participants. DNA was suspended in Tris-EDTA (TE) buffer, pH 8, and stored at -20 °C until further investigation.

Polymerase chain reaction: The promoter, 5' UTR region of *VEGF*, and the intron4 region of *eNOS* were PCR amplified and conditions adopted as per previous protocol [22,23]. The *eNOS* polymorphic genotypes were assessed directly through the Amplification Refraction Mutation System (ARMS) PCR and the products were electrophoresed on 1.5% agarose gel. Two types of PCR products were obtained, a 420 bp band which indicates five repeats of the 27 bp allele (b allele), and a 393 bp band that indicates four repeats of the 27 bp allele (a allele).

Restriction fragment length polymorphism analysis: The PCR products were restriction digested using 5 units of enzymes *Fnu4HI* for -T(-1498)C, *DdeI* for -G(-1190)A and C(-7)T, and *BsmFI* for -C(-634)G (New England Biolabs, Beverly, MA) in a 10 µl setup and visualized on 3% agarose gel electrophoresis to detect the reported polymorphisms in *VEGF*.

Statistical analysis: Allele frequencies were calculated from the observed numbers of genotypes. Differences in the allele frequencies between the groups were tested by Pearson's χ^2 test and Fisher's exact test. Monte-Carlo methods were used to confirm the estimations of haplotype differences due to concern over sparse contingency tables. Continuous clinical data were compared by unpaired Student's t-test. Multiple logistic regression analysis was performed to assess the role of the *VEGF* genotype and other variables, including age, sex,

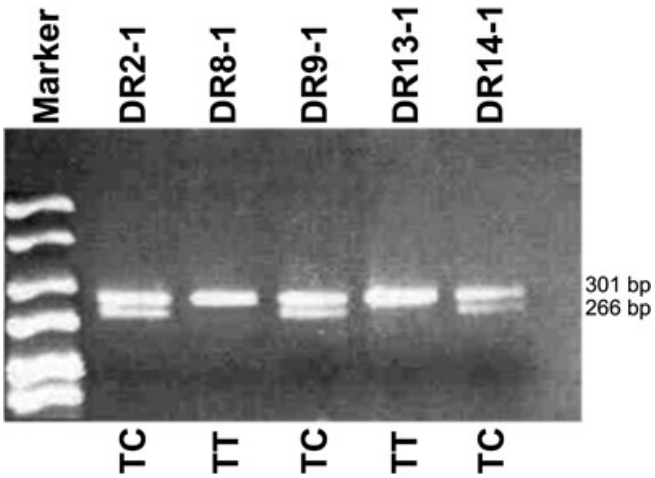

Figure 3. *Fnu*4HI restriction analysis for T(-1498)C polymorphism. Restriction-fragment length polymorphism analysis showing the restriction fragments of the *VEGF* polymorphism, T(-1498)C, which created the *Fnu*4HI restriction site. The molecular marker is a 100 bp ladder.

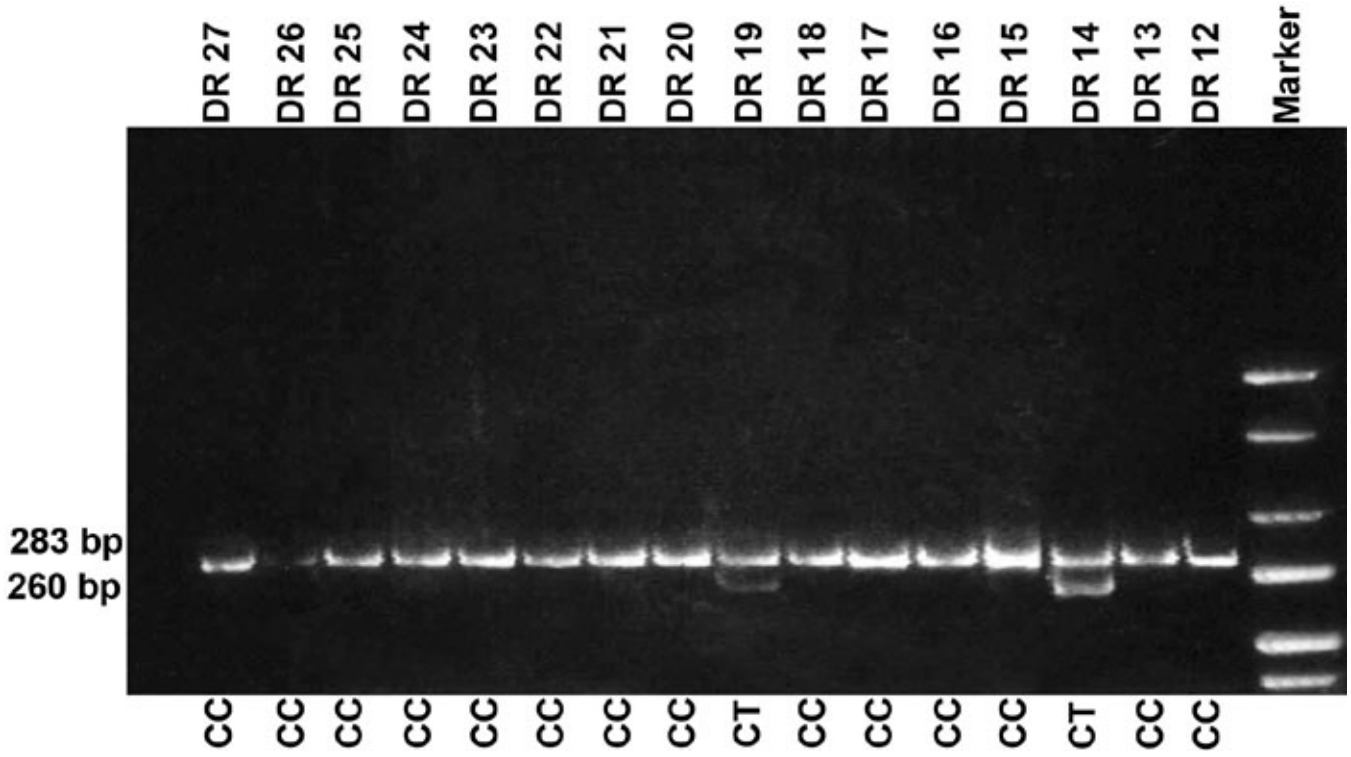

Figure 2. Detection of *VEGF* C(-7)T polymorphism by RFLP analysis. The genotypes associated with the C(-7)T polymorphism were restriction digested with *Dde*I, and the fragments were visualized on a 3% agarose gel. Fragment size for each allele given in Table 2. The 283 bp band indicates the C allele, the 260 bp band indicates the T allele, and the 283 bp and 260 bp bands indicate the CT genotype. Based on this banding pattern the genotype is provided below each well. The molecular marker is a *Puc*19 ladder.

and duration of diabetes. Stata 8.1 (Stata Corporation, College station, TX) was used to do the statistical analyses.

RESULTS

We recruited, 120 DR and 90 DWR patients for genetic analysis. Their clinical characteristics are given in Table 1. Restriction-fragment length polymorphism (RFLP) analysis was performed to evaluate the genotypes of polymorphisms C(-7)T and C(-634)G in the 5' UTR and T(-1498)C and G(-1190)A in the promoter region of *VEGF*. The alleles -634G (Figure 1), -7T (Figure 2), and -1498C (Figure 3) resulted in gains of *Fnu4HI*, *BsmFI*, and *DdeI* restriction sites, respectively. The PCR products and allele variations of *VEGF* are presented in Table 2. A comparison of genotype and allele frequencies between both the DWR and DR group revealed that genotype distribution of C(-7)T, T(-1498)C, and C(-634)G polymorphisms were statistically significant in the DR group (Table 3). In addition, the allele distribution also revealed that the C allele of the -1498 region (p=0.0001) and T allele of the -7 (p=0.002) region in *VEGF* were significantly increased in the DR group (Table 4). However, calculated odds ratios estimating the presence of CT genotype in C(-7)T region (OR=4.17, 95% CI: 1.90-9.18, p=0.0001), the TC genotype in T(-1498)C region (OR=4.37, 95% CI: 2.44-7.84, p=0.0001) and the CG genotype in C(-634)G region (OR=2.3, 95% CI: 1.27-4.45, p=0.008) were significantly higher in the DR group compared with the DWR group. The power of the study calculated to detect the odds ratio of 4.37 for TC genotype in VEGF T(-1498)C between DR and DWR is more than 80%.

Multiple logistic regression analysis was performed to analyze the relevance of selected parameters (age, sex, dura-

Table 2. Polymerase chain reaction RFLP analysis of *VEGF*

Region	PCR product size (bp)	Restriction enzyme	Restriction fragment sizes (bp)
T(-1498)C	301	Fnu4HI	301(T), 35+266(C)
G(-1190)A	592	DdeI	404+188(G), 404+145+43(A)
C(-634)	470	BsmFI	470(C), 196+274(G)
C(-7)T	283	DdeI	283(C), 20+263(T)

The size of the PCR product and the restriction enzymes for each polymorphism and their resulting restriction fragments are listed.

Table 3. Genotype distribution of *VEGF* and *eNOS* polymorphisms in DR and DWR

Gene/region	Genotype	Diabetic retinopathy (n=120)	Diabetic without retinopathy (n=90)	p
VEGF T(-1498)C	TT	30.0	67.8	0.0001
	TC	67.5	32.2	
	CC	2.5	-	
VEGF G(-1190)A	GG	55.0	61.1	0.500
	GA	25.8	25.6	
	AA	19.2	13.3	
VEGF C(-634)G	CC	17.5	30.0	0.021
	CG	80.8	64.4	
	GG	1.7	5.6	
VEGF C(-7)T	CC	68.3	88.9	0.001
	CT	31.7	10.0	
	TT	-	1.1	
eNOS	b/b	71.7	76.7	0.607
	b/a	22.5	20.0	
	a/a	5.8	3.3	

The genotype distribution for polymorphisms were compared between diabetic patients with and without retinopathy using the χ^2 test. Data are given as percentages.

Table 4. Allele distribution of *VEGF* and *eNOS* polymorphisms in DR and DWR

Gene/region	Allele	Diabetic retinopathy (n=240)	Diabetic without retinopathy (n=180)	Odds ratio (95% CI)	p
VEGF T(-1498)C	T C	63.75 36.25	83.33 16.67	2.84 (1.75-4.61)	0.0001
VEGF G(-1190)A	G A	67.92 32.08	73.89 26.11	1.33 (0.86-2.05)	0.18
VEGF C(-634)G	C G	58.33 41.67	61.11 38.89	1.12 (0.75-1.66)	0.56
VEGF C(-7)T	C T	84.17 15.83	93.89 6.11	2.89 (1.42-5.88)	0.002
eNOS intron4	b a	16.25 83.75	14.44 85.56	0.87 (0.50-1.49)	0.61

The allele distribution of each polymorphism was compared between diabetic patients with and without retinopathy. The frequencies of alleles are given in percent.

Table 5. Relation of gender, age, duration, and genotypes with type 2 diabetic retinopathy

Variable	Odds ratio (95% CI)	p
Female	0.86 (0.36- 2.04)	0.737
Age	0.94 (0.90- 0.99)	0.024
Duration	0.92 (0.87- 0.98)	0.009
VEGF C(-634)G/CC genotype	2.09 (0.20-21.40)	0.534
VEGF C(-634)G/CG genotype	2.38 (0.26-21.55)	0.440
VEGF C(-7)T/CT genotype	4.28 (1.68-10.92)	0.002
VEGF T(-1498)C/TC genotype	4.77 (2.28- 9.94)	0.001

Table shows adjusted odds ratios with 95% confidence intervals (95% CI) obtained from multiple logistic regression. Models were adjusted by logistic regression analysis for the association with retinopathy among type 2 diabetes patients.

Table 6. Distribution of probable haplotypes of *VEGF* polymorphisms in DR and DWR

Position				Diabetic retinopathy (n=240)	Diabetic without retinopathy (n=180)	p
-1498	-1190	-634	-7			
C	A	C	C	4.2	0.5	0.012
C	A	G	C	22.1	6.1	0.000
C	G	G	T	1.7	0.5	0.398
T	A	C	C	8.7	5.6	0.079
T	G	C	C	43.3	46.7	0.179
T	G	G	C	8.3	18.9	0.081
Other haplotypes				11.7	21.7	0.262

Haplotypes were compared between diabetic retinopathy (DR) and diabetic without retinopathy (DWR) using Monte Carlo techniques. Data are given in percent. The "Other haplotypes" line combines haplotypes CACT, CAGT, CGCC, CGCT, CGGC, TAGC, TGCT, and TGGT for comparison between DR and DWR.

tion of diabetes, and genotypes of *VEGF* polymorphisms) as given in Table 5. Age, duration of diabetes, and -7CT, -1498TC genotypes proved to be significant predictors of DR (p=0.024, p=0.009, p=0.002, and p=0.001, respectively). Based on this logistic regression analysis, the duration is one of the significant predictors of diabetes for DR. We have divided the duration of diabetes into two groups: lower (less than or equal to 15 years) and higher (>15 years) duration. If the diabetes duration is less than or equal to 15 years, the risk of developing DR is 1.91 times higher when compared with DWR and it was statistically significant (p=0.02).

As shown in Table 6, six major haplotypes were estimated and each estimate was compared between DR and DWR using the Monte Carlo simulation test. We found that the -1498C/-1190A/-634C/-7C (p=0.012) and -1498C/-1190A/-634G/-7C (p=0.000) haplotypes were significantly increased in patients with DR. The significance of the haplotype is due to *VEGF* T(-1498)C, C(-634)G, and C(-7)T polymorphisms. Taken together, the data suggest that the *VEGF* polymorphisms are associated with DR.

We have also identified one reported polymorphism (intron 4) in *eNOS* in patients with DR and DWR, but the *eNOS* polymorphism did not differ significantly between the two study groups (data not shown). This study shows *VEGF* polymorphisms have a high significant association with DR in the Indian population.

DISCUSSION

As VEGF is involved in the process of new blood vessel formation, it seems to be a potential candidate gene for DR. In this study, we have focused on the 5' UTR, promoter region of *VEGF* for genetic analysis as it has been shown to be highly polymorphic. In addition, most of the hypoxia responsive elements are present in this region and retinal hypoxia has been observed in DR. During hypoxia, hypoxia-inducible factors bind to the hypoxia-response elements and induce the expression of *VEGF* [24-26], which leads to the stimulation of angiogenesis. The polymorphisms in this gene have been studied in different ethnic groups but not in the Indian population. Hence we chose to examine *VEGF* for possible association with DR.

In the *VEGF* promoter and 5' UTR region, three polymorphisms were identified (C(-7)T, T(-1498)C, and C(-634)G). Comparison of the allele frequencies of these *VEGF* polymorphisms and their genotype combinations, haplotype frequencies between DR and DWR groups revealed significant results. In the Japanese population Takuya Awata et al. [22] identified C(-7)T, T(-1498)C, G(-1154)A, C(-634)G, and G(-1190)A polymorphisms in *VEGF*. They identified the CC genotype of the C(-634)G region to be significantly associated with DR (OR=3.20, 95% CI: 1.45-7.05, p=0.0046), whereas the CG genotype of the C(-634)G region was significantly associated with DR (OR=2.33, 95% CI: 1.24-4.36, p=0.008) in the Indian population. In addition to that, other genotypes of CT in the C(-7)T region and TC in the T(-1498)C region of *VEGF* were also found to be statistically significant in DR, whereas these genotypes were not reported to be significant in the Japanese population. Multiple logistic regression revealed nongenetic parameters such as diabetes mellitus duration and age, and genetic parameters like T(-1498)C and C(-7)T polymorphisms in *VEGF* as significant predictors of DR.

Comparison of distribution of genotype combinations between DR and DWR groups revealed significant differences. Combinations largely consisting of a majority of alleles in the -7C/T, -1498T/C, and -634C/G positions were found to be significantly associated with DR.

When the results obtained from our study were analyzed, the frequencies of heterozygous genotypes of *VEGF* C(-7)T, T(-1498)C, and C(-634)G were found to be significantly higher in patients with DR compared with DWR. The other genotypes in *VEGF* did not differ significantly between the two study groups. We suggest that the three heterozygous genotypes may be the high risk factors for DR. The association of polymorphism related to DR still remains unclear. Therefore further studies are required to conclusively establish the gene link between the genotype and DR.

The *eNOS4b/b* genotype was significantly higher in severe DR in caucasian populations [23]. In our study the genotypes of *eNOS* did not differ significantly between the DR and DWR study groups. Our data suggest that the *VEGF* polymorphisms might be a major risk factor in the development of DR in the Indian population.

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