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Research Article

Vascular Endothelial Growth Factor-A (*VEGFA*) Gene Polymorphisms and Genetic Predisposition of Retinopathy in Type 2 Diabetes Patients of India

Dhara Nareshkumar Jajal[1] and Kiran Kalia[1,2]

[1]Lab # 103B, B. R. D. School of Biosciences, Sardar Patel University,
Vallabh Vidyanagar – 388 120, Gujarat, India
[2]National Institute of Pharmaceutical Education & Research – Ahmedabad,
Nr. Palaj Village, Gandhinagar - 382355, Gujarat, India
[*]Corresponding author: Kiran Kalia, Email: kirankalia@gmail.com, director@niperahm.ac.in,
Tel: +91-9714618573, +91-9824335881; Fax: +91-79- 27450449

ABSTRACT:

Purpose: Vascular Endothelial Growth Factor - A (VEGFA) promotes angiogenesis and its role in the pathology of diabetic retinopathy (DR) is well documented. Although the polymorphisms in VEGFA gene have been shown to increase the risk of DR development and progression in various ethnicities, few studies have been carried out targeting the intronic SNPs. Therefore, the main purpose of present study was to assess the genetic predisposition of DR and proliferative DR (PDR) attributed by three intronic polymorphisms of VEGFA gene among type 2 diabetes (T2D) patients.

Method: We enrolled total 351 unrelated individuals [93 healthy controls (HC), 110 T2D patients without retinopathy (DWR) and 148 T2D patients with retinopathy (DR)] from the western region of India. Genotyping of rs833069, rs2146323, and rs3025021 SNPs was performed by PCR-RFLP.

Results: The AA genotype in a co-dominant model and minor allele (A) of rs2146323 was significantly high in PDR patients when compared to DWR patients ($p = 0.003$ and $p = 0.010$, respectively). However, the SNP was not significantly associated with DR when compared to HC or DWR individuals on applying multivariate logistic regression ($p = 0.142$ and $p = 0.045$, correspondingly). We did not observe significant variation in the distribution of rs833069 and rs3025021 polymorphisms among the study groups. Our data suggested rs833069 and rs2146323 SNPs were in linkage disequilibrium ($D'=0.947$), and ACC of the observed haplotypes showed a significant inverse association with PDR ($p = 0.001$).

Conclusion: Our study suggested that minor homozygous genotype of rs2146323 conferred two-fold risk to develop PDR in the targeted Indian ethnicity. Further studies in larger population would help in confirming the association substantially.

Keywords: VEGFA gene, Diabetic retinopathy, Intronic SNPs, Indian population

[I] INTRODUCTION

Diabetic retinopathy (DR) - a microvascular complication of diabetes, is a well-recognized consequence of long-standing and poorly controlled hyperglycemia. It is estimated that DR affects from 12 to 30% of the diabetic population in India [1]. DR is chiefly characterized by retinal microaneurysms, vascular exudations of proteins in retina or macula, and subsequent neovascular proliferation accompanied by vitreous hemorrhage [2]. The precise molecular mechanism of DR is poorly understood due to its multifaceted pathogenesis. Moreover, the conventional risk

factors like prolonged hyperglycemia and oxidative stress do not entirely explain the etiology of DR [3,4].

Previous studies have evidently shown the ethnicity specific high prevalence and heritability of DR, including an Indian population [5-8]. Thus, genetic factors are well established in the predisposition of DR, and their identification is an area of substantial research for developing screening algorithms for early detection of DR. Several studies have reported potential genes associated with DR prevalence that evidently includes vascular endothelial growth factor - A (VEGFA).

It is an endothelial cell specific chemokine that mediates angiogenesis and has been known to play a pivotal role in the pathogenesis of DR. VEGFA expression is stimulated by oxidative stress produced in diabetic retina. As a result, markedly elevated levels of VEGFA have been reported in ocular fluid of patients with DR [9-11].

During the DR progression, VEGFA mediates breakdown of blood-retinal barrier and ischemia-induced neovascularization that is hyperpermeable, the condition known as proliferative diabetic retinopathy (PDR) [10,11]. It can eventually lead to retinal detachment and ultimately to blindness if left untreated. However, therapeutic administration of VEGFA antagonists has demonstrated reduced retinal permeability and neovascularization [12,13].

The human VEGFA gene is mapped on chromosome region, 6p21.3, which spans to approximately 18kb including promoter region and it is highly polymorphic.

The VEGFA consists of eight exons, and its alternative splicing refers to the two VEGFA protein families, namely, pro-angiogenic and anti-angiogenic [14]. So far, the most investigations have analyzed polymorphisms in the 5′ untranslated region and promoter of the VEGFA for finding their influence on the susceptibility of DR and/or PDR.

Nevertheless, fewer studies have taken the intronic variations of VEGFA into consideration for the same. The previous studies targeting intronic SNPs of VEGFA gene that may increase the DR occurrence among type 1 and type 2 diabetic (T2D) patients have shown widely conflicting results [15-21].

Moreover, genetic association studies require trans-racial approach since ethnicity influences the allele frequencies of SNPs and their linkage disequilibria.

Therefore, we aimed to evaluate the common intronic SNPs of VEGFA gene (rs833069, and rs2146323 of intron 2, and rs3025021 of intron 6) for predisposing our distinct Indian T2D population to DR and PDR;

which were not investigated earlier in the Indian population as per our knowledge. The studied SNPs are illustrated on VEGFA gene map in figure 1.

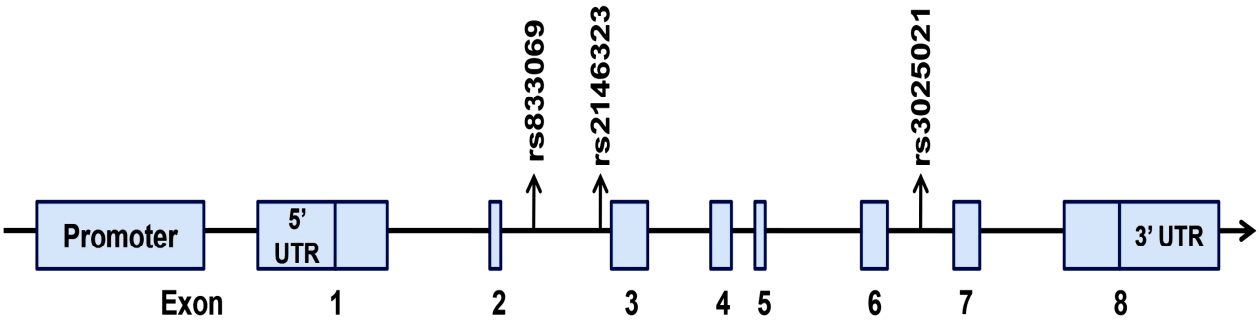

Figure: 1. *VEGFA* gene map showing the locations of the three SNPs analyzed in the study

[II] MATERIALS AND METHODS

2.1. Participants

The present study was approved by Human Research Ethics Committee of Pramukh Swami Medical College and Hospital, Karamsad, Gujarat. The study was conducted as per the principles embodied in the Declaration of Helsinki. We made all participants acquainted with the research and obtained informed consents before sample collection. In our cross-sectional study, we incorporated 258 T2D patients who had attended Pramukh Swami Medical College and Hospital, Karamsad, Gujarat, from March 2013 to May 2015. The study comprised of total four groups:

1. Healthy controls (HC, n = 93)
2. T2D patients without retinopathy (DWR, n = 110)
3. T2D patients with retinopathy (DR, n = 148)
4. T2D patients with proliferative retinopathy – a subgroup of DR (PDR, n = 62)

The treating physician diagnosed the patients with T2D as per the American Diabetes Association (ADA) guidelines. All the patients and controls were undergone visual acuity and fundus examination through dilated pupils. An expert ophthalmologist diagnosed them for DR grading according to Diabetic Retinopathy Disease Severity Scale based on Early Treatment Diabetic Retinopathy Study (ETDRS) [22].

The HC group was characterized by normal blood pressure, normal fundus test, the absence of parental diabetes history and any other diseased condition. They were volunteers, blood donors, or relatives to the patients that visited the hospital. We interviewed all subjects through a structured questionnaire, and their clinical characteristics were noted.

All subjects belonged to the western part of India, more precisely Gujarat, and hence were from the same geographical area and culture. The patients with malignancy, inflammatory diseases, other genetic diseases, amputations, end stage renal disease, history of cardiac stroke or bypass surgery, paralysis were excluded from the study.

2.2. Biochemical Analysis

Fasting blood samples were collected in EDTA-coated Vacutainers (BD Biosciences) and transported to the laboratory at 4 °C. The fasting plasma glucose (FPG) and serum creatinine levels were measured by GOD-POD and Jaffe's reaction based kits, respectively. The glycated hemoglobin (GHb) was estimated by a conventional thiobarbituric acid method.

2.3. Genotyping of the SNPs

The DNA extraction was carried out using QIAamp® DNA Blood kit (Qiagen) by following the manufacturer's protocol. The DNA samples with absorption at 260 nm/ 280 nm $\geq$ 1.8 were used for genotyping of the SNPs. We performed PCR-RFLP to genotype rs833069 T>C, rs2146323 C>A, and rs3025021 T>C SNPs in all participants. The specific PCR primers were designed by the Primer-Blast tool of NCBI. All samples were amplified in a 25 ul reaction mixture using Applied Biosystems® Veriti® thermal cycler.

Each reaction contained 12.5 ul Taq 2x master mix (New England BioLabs), 400-450 nM forward and reverse primers (Eurofins), 40 ng DNA template, and H2O to make up the volume. Table 1 is representing the primers used in the study, their respective Tm, and product length. The PCR cycling conditions were: denaturing at 95ºC for 1 min, 1 cycle; denaturing at 94ºC for 30 s, annealing at the pertinent Tm for 20 s, extension at 68ºC for 30 s, 30 cycles and a final extension at 68ºC for 5 min. The 2.5% agarose gel was run to verify the specific PCR products visualized by ethidium bromide staining.

The restriction enzymes were selected by an online application, NEBcutter. The selected restriction enzymes and their corresponding fragment pattern to genotype the SNPs are summarized in Table 1.

SNP	Primer	Tm	Product Size	RE	Fragment Pattern
rs833069	F: 5'GTTCACAGCACCCGAACATA3' R: 5'GAACAGCGGAGAGTCCTCAC3'	58 ºC	358 bp	BseRI	C allele: 358 bp T allele: 274 bp & 84 bp
rs2146323	F: 5'GTCTCGATTGGATGGCAGTA3' R: 5'CCCATACTCAGACTGTCCTCT3'	57 ºC	384 bp	MluCI	C allele: 384 bp A allele: 224 bp & 160 bp
rs3025021	F: 5'TTCCACCAAGGTGGGCTAAA3' R: 5'CTGCTCACCCAACTGGTTTC3'	60 ºC	352 bp	NciI	T allele: 333 bp & 19 bp C allele: 260 bp, 73 bp & 19 bp

Table 1: Primers, their corresponding Tm and product size, and restriction enzymes with their fragment pattern used to genotype the SNPs

The restriction digestion was done in a 25 ul reaction containing 1x Cutsmart buffer (New England BioLabs), 10 ul of PCR product and 2 U of specific restriction enzyme (New England BioLabs). The reactions for all SNPs were incubated at 37ºC for 6-8 h for complete restriction digestion.

The digested PCR products were run on a 3% agarose gel, and the genotyping was done according to the visualized fragments.

2.4. Statistical Analysis

The study used the R statistical package to perform the statistical analysis of the data. Baseline demographic and biochemical characteristics were compared between study groups by Kruskal-Wallis test and one-way ANOVA for non-parametric and parametric continuous variables, respectively.

The assessment of categorical covariates was done by two-tailed Fisher's exact test. The chi-square test was performed to examine the Hardy–Weinberg equilibrium (HWE) at the individual polymorphic locus.

The p-value less than 0.05 was considered statistically significant. Univariate and multivariate logistic regression analyses were carried out to analyze the distribution of SNPs among the study groups. Boneferroni correction was applied for multiple testing for which statistically significant cutoff p-value was less than 0.016. Additionally, odds ratios (OR) with 95% confidence intervals (CI) were estimated to identify the risk of DR and PDR. Linkage disequilibrium (LD) between SNPs and association of observed haplotypes were determined by SHEsis software [23,24].

[III] RESULTS

We studied total 351 individuals, out of which 110 T2D patients did not show DR characteristics that were designated as DWR group. On the other hand, 148 patients were diagnosed for DR, out of which 62 patients showed characteristics of PDR at least in one eye. Moreover, more than half (65%) of the DR patients were diagnosed with grade 4 or above on ETDRS severity scale.

3.1. Demographic, Clinical and Biochemical Findings of the Study Groups

The demographic, clinical and biochemical details of the studied groups are given in Table 2. The DR patients were significantly older in age than HC and DWR subjects, whereas, when the age at the onset of T2D was considered, it did not show the variation among HC, DWR and DR groups (mean age 48.3, 47.8, and 49.8, correspondingly). Systolic blood pressure was significantly higher in T2D patients than in HC, whereas, only DR group presented significant high diastolic blood pressure than the healthy HC group. We observed that 31.8% and 61.5% of the DR individuals were correspondingly having diabetes for ≤ 5 years and ≤ 10 years, indicating early onset of retinopathy in the studied population. Conversely, the other confounders like the presence of hypertension, duration of diabetes, family history of diabetes and habits were non-significantly different between both the groups of T2D. As shown in Table 2, all the biochemical parameters were found to be significantly elevated in DR patients when compared to DWR and HC participants. Further, DWR subjects showed apparent increased levels of FPG and GHb than HC individuals ($P < 0.0001$).

Table 2: Clinical and biochemical characteristics of the study groups

Parameters	HC	DWR	DR	P value
Number	93	110	148	
Age (years)	48.3 ± 11.5	55.3 ± 9.0	59.1 ± 7.4	a*** b*** c**
Gender [n (%)]				
Male	53 (57.0)	66 (60.0)	88 (59.5)	$(a\ b\ c)^{NS}$
Female	40 (43.0)	44 (40.0)	60 (40.5)	
BMI (kg/m^2)	24.3 ± 3.9	26.4 ± 4.0	24.9 ± 4.0	a** b^{NS} c*
Hypertensive Patients [n (%)]		59 (53.6)	95 (64.2)	C^{NS}
SBP (mm hg)	122.0 ± 7.4	131.9 ± 16.4	132.7 ± 14.1	a*** b*** c^{NS}
DBP (mm hg)	79.5 ± 5.6	80.9 ± 9.2	82.6 ± 9.2	a^{NS} b* c^{NS}
Duration of diabetes (years)	-	7.5 ± 3.3	8.9 ± 5.4	C^{NS}
Unknown	-	0	7	
Hypoglycaemic agents [n (%)]				
Oral Hypoglycaemic agents	-	101 (91.8)	115 (77.7)	C*
Insulin	-	3 (2.7)	13 (8.8)	
Oral + Insulin	-	4 (3.7)	14 (9.5)	
No medications	-	2 (1.8)	6 (4.0)	
Family History of Diabetes [n (%)]		51 (49.0)	66 (46.2)	
Mother	-	29	29	C^{NS}
Father	-	25	20	
Siblings	-	15	35	
Unknown	-	6	5	
Habits [n (%)]		43 (39.1)	54 (36.5)	
Tobacco chewing/ sniffing	-	26/7	29/2	C^{NS}
Smoking	-	13	17	
Occasional/ former Habituate	-	3/0	8/2	
Fasting Plasma Glucose (mg/dl)	84.5 ± 7.2	130.2 ± 20.6	138.9 ± 26.4	a*** b*** c*
Glycated Hemoglobin (%)	6.31 ± 0.68	8.99 ± 0.71	9.57 ± 1.04	a*** b*** c***
Serum Creatinine (mg/dl)	0.99 ± 0.26	1.01 ± 0.28	1.12 ± 0.31	a^{NS} b** c*

Data are shown as mean ± SD wherever applicable. a is comparison between HC and DWR groups, b is comparison between HC and DR groups, and c is comparison between DWR and DR. * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$, ^{NS} P value non significant. BMI, Body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure.

Table 3: Genotypic and Allelic distributions of VEGF gene polymorphisms among studied groups

Genotype/ Allele	HC [n (%)]	DWR [n (%)]	DR [n (%)]	PDR [n (%)]
rs833069 (3596 T>C)				
TT	53 (57.0)	64 (58.2)	93 (62.8)	36 (58.1)
TC	37 (39.8)	42 (38.2)	49 (33.1)	23 (37.1)
CC	3 (3.2)	4 (3.6)	6 (4.1)	3 (4.8)
T Allele	143 (76.9)	170 (77.3)	235 (79.4)	95 (76.6)
C Allele	43 (23.1)	50 (22.7)	61 (20.6)	29 (23.4)
rs2146323 (6112C>A)				
CC	42 (45.2)	49 (44.5)	57 (38.5)	19 (30.6)
CA	50 (53.7)	54 (49.1)	69 (46.6)	30 (48.4)
AA	1 (1.1)	7 (6.4)	22 (14.9)	13 (21.0)
C Allele	134 (72.0)	152 (69.1)	183 (61.8)	68 (54.8)
A Allele	52 (28.0)	68 (30.9)	113 (38.2)	56 (45.2)
rs3025021 (10180C>T)				
CC	57 (61.3)	54 (49.1)	79 (53.4)	31 (50.0)
CT	31 (33.3)	50 (45.5)	53 (35.8)	21 (33.9)
TT	5 (5.4)	6 (5.4)	16 (10.8)	10 (16.1)
C Allele	145 (78.0)	158 (71.8)	211 (71.3)	83 (66.9)
T Allele	41 (22.0)	62 (28.2)	85 (28.7)	41 (33.1)

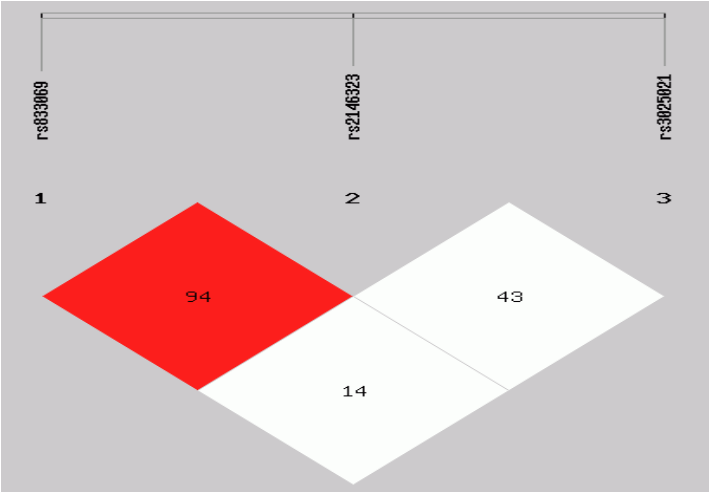

Figure: 2. Linkage disequilibrium (LD) plot of the studied SNPs**:** LD is displayed in the boxes as the pairwise D' value multiplied by 100. The magnitude and significance of the pairwise LD is represented with a red-to-white gradient reflecting higher-to-lower LD values.

3.2. Association of the SNPs with DR

The analyzed SNPs were in Hardy–Weinberg equilibrium in all study groups. Table 3 demonstrates the genotype and allele frequencies of targeted SNPs in the study groups. The univariate analysis of the SNPs in a co-dominant model indicated a significant correlation of AA genotype of rs2146323 with DR when compared to HC (p = 0.0004, OR 2.22, 95% CI 1.44 - 3.43),

but not when compared to DWR (p = 0.033, OR 1.25, 95% CI 1.02 - 1.53) **[Table 4]**.

However, in comparison with DWR patients, PDR patients were having the significant high frequency of AA genotype for univariate as well as multivariate logistic regression analysis (p = 0.002, OR 2.10, 95% CI 1.31 -3.36 and p = 0.003, OR 1.95, 95% CI 1.27 -2.99, respectively). We observed that out of 22 DR patients harboring AA genotype, correspondingly 13 (63.6%) and 6 (22.7%) patients were diagnosed with PDR and severe nonproliferative diabetic retinopathy (NPDR). Moreover, the frequency of AA genotype of the SNP was non-significantly higher among DWR patients in comparison with HC subjects **[Table 4]**. Further, the multivariate analysis for rs2142363 suggested that apart from AA genotype, FBG, serum creatinine, and age were the other confounding factors responsible significantly for developing PDR in the targated population.

As shown in Table 4, the genotype and allele distributions for the other two SNPs, rs833069 and rs3025021 did not vary significantly among the studied groups. Hence, we report a lack of correlation of both the SNPs with DR or PDR in the targeted Indian ethnicity. The LD analysis revealed the strong linkage disequilibrium between rs833069 and rs2142363 having D' value of 0.947 **[Figure 2]**.

The haplotype ACC (rs833069, rs2142363, rs3025021) was inversely associated with PDR group in comparison to DWR group (Haplotype frequency 0.14 vs 0.31, p = 0.001); suggesting ACC as a protective haplotype for severe DR. Conversely, none of the observed haplotypes indicated a significant association with DR.

[IV] DISCUSSION

The present study examined the association of the three intronic SNPs of VEGFA gene with the prevalence of DR and PDR in the Indian subset. One of the frequently studied SNP among them was rs2146323 (C/A) that is located in intron 2 of VEGFA gene. Previously, the SNP was genotyped in Caucasian, Finish, Chinese, and Australian populations for assessing its correlation with DR and/or PDR **[Table 5]**.

In our study, AA genotype of rs2146323 attributed two-fold risk of developing PDR among T2D patients (p = 0.003) which was not observed for DR. Further, the identified risk genotype was significantly associated with the severity of DR (i.e. PDR and severe NPDR patients) (p = 0.0001). On the contrary, the case-control studies in Australian, and Finish populations did not show an association with the same [15, 18].

Opposite to our findings, the C allele of rs2146323 was suggested as a potent risk factor for the development of PDR among type 1 and 2 diabetic Caucasians [17]. In partial affirmation with our results, A allele of rs20146323 was found to be associated with DR in the later study of Yang et al. (2014) (P = 0.004) and not in their earlier study; suggesting sample size play a crucial role in the risk assessment [20, 21].

Table 4: Comparisons of Genotypic and Allelic distribution of VEGF gene polymorphisms between the study groups

SNP	Comparison	HC vs DWR		HC vs DR		DWR vs DR		DWR vs PDR	
		P value	**OR (95% CI)**	**P value**	**OR (95% CI)**	**P value**	**OR (95% CI)**	**P value**	**OR (95% CI)**
	TT vs TC	0.834	0.98 (0.85 -1.14)	0.313	0.87 (0.67-1.13)	0.411	0.95(0.83 -1.08)	0.937	0.99 (0.73 -1.34)
rs833069		0.112*	0.96 (0.91 -1.01)*	0.182*	0.92 (0.82 -1.04)*	0.156*	0.91 (0.80 -1.04)*	0.579*	0.92 (0.70 -1.22)*
(3596 T>C)	TT vs CC	0.901	1.02 (0.70 -1.50)	0.86	1.06 (0.55 -2.05)	0.962	1.01 (0.73-1.38)	0.718	1.15 (0.55-2.41)
		0.351*	0.94 (0.82 -1.08)*	0.965*	1.01 (0.75 -1.35)*	0.402*	0.88 (0.64 -1.19)*	0.905*	0.96 (0.49 -1.87)*
	CC vs CA	0.787	0.98 (0.85 - 1.13)	0.867	1.02 (0.79 -1.33)	0.722	1.02 (0.90 -1.16)	0.313	1.17 (0.86 -1.58)
rs2146323		0.914*	0.99 (0.95 - 1.05)*	0.580*	0.97 (0.86 -1.09)*	0.926*	1.01 (0.89 - 1.14)*	0.468*	1.11 (0.84 -1.45)*
(6112C>A)	CC vs AA	0.068	1.40 (0.98 - 2.01)	0.0004	2.22 (1.44 -3.43)	0.033	1.25 (1.02 -1.53)	**0.002**	**2.10 (1.31 -3.36)**
		0.110*	1.11 (0.98 - 1.27)*	0.142*	1.17 (0.95 -1.44)*	0.045*	1.23 (1.01 -1.50)*	**0.003***	**1.95 (1.27 -2.99)***
	CC vs CT	0.074	1.14 (0.99 - 1.31)	0.436	1.11 (0.85 -1.46)	0.222	0.92 (0.81 -1.05)	0.369	0.87 (0.65 - 1.18)
rs3025021		0.359*	0.98 (0.93 - 1.03)*	0.398*	1.05 (0.93 -1.19)*	0.382*	0.95 (0.83 - 1.07)*	0.496*	0.91 (0.69 -1.20)*
(10180C>T)	CC vs TT	0.708	1.06 (0.78 - 1.44)	0.123	1.44 (0.91 -2.28)	0.242	1.14 (0.91 -1.43)	0.046	1.68 (1.013 -2.80)
		0.403*	0.85 (0.64 - 1.12)*	0.676*	1.04 (0.85 -1.28)*	0.551*	1.07 (0.85 -1.34)*	0.571*	1.15 (0.71 -1.88)*

*indicates adjusted P value or adjusted odds ratio with 95% confidence interval where both represent data after adjustment for covariates including age, diabetes duration, family history of diabetes, hypertension, glycated hemoglobin (GHb), fasting plasma glucose and serum creatinine levels.

Besides cross-sectional studies, longitudinal studies have been carried out in North America and Japan which showed the earliest evidence that A allele of rs2146323 polymorphism influences the progression of DR in type 1 diabetes patients [16, 19]. Both the studies have included rs3025021, where discrepantly Al-Kateb et al. found it a potential risk factor for severe DR. Interestingly, the Asian ethnicities did not identify the role of rs3025021 in DR development so far **[Table 5]**. Similar to the present study, Yang et al. have not found the significant difference in the genotypic and allelic distributions of rs833069 between DWR and DR groups [20, 21]. Though our some results were similar to the Asian ethnicities, the discrepancy was observed in the genotypic and allelic distributions among DWR and DR patients as shown in Table 5.

SNP	Population	Genotype Frequency (homozygous major/ heterozygous/ homozygous minor)		Minor Allele Frequency		Type of Diabetes	Association	Sample size DWR/DR	Reference
		DWR	DR	DWR	DR				
rs833069 (TT/TC/CC)	Chinese	27.3/51.8/20.9	27.1/55/17.8	46.8	45.3	2	DWR vs DR	139/129	[20]
	Chinese	30.5/51.8/20.6	32.6/48.8/18.6	46.5	43	2	DWR vs DR	282/215	[21]
	Present Study	58.2/38.2/3.6	62.8/33.1/4.1	22.7	20.6	2	DWR vs DR, PDR	110/148	
rs2146323 (CC/CA/AA)	Caucasian DCCT/EDIC cohort	-	-	-	35.7	1	DWR vs Severe DR*	1362	[16]
	Caucasians of Northern Europe	42.6/36.1/21.3	42.2/57.8/0	39.4	28.9	1 & 2	DWR vs PDR**	61/45	[17]
	Australian	47/38/15	31/59/11	34.0	40.5	1	DWR vs Blinding DR	93/75	[15]
	Australian	47/43/10	39/50/10	31.5	35.0	2	DWR vs Blinding DR	182/137	[15]
	Japanese	-	55.2/ 37.9/ 6.9	-	25.9	1	DWR vs Severe NPDR*	174	[19]
	Finish	41/45/13	37/48/15	36	39	1 & 2	DWR vs DR	98/131	[18]
	Chinese	63.8/30.4/5.8	52.3/35.9/11.7	21.0	29.7	2	DWR vs DR	168/98	[20]
	Chinese	60.5/33.7/5.8	52.3/33.6/14	22.6	30.8	2	DWR vs DR*	276/214	[21]
	Present Study	44.5/49.1/6.4	38.5/46.6/14.9	30.9	38.2	2	DWR vs PDR**	110/148	
rs3025021 (CC/CT/TT)	Caucasian DCCT/EDIC cohort	-	-	-	32.3	1	DWR vs Severe DR**	1341	[16]
	Japanese	-	75.3/ 22.4/ 2.3	-	13.5	1	DWR vs Mild NPDR, Severe NPDR, PDR	174	[19]
	Australian	39/48/13	39/50/11	37	36	1	DWR vs Blinding DR	94/76	[15]
	Australian	48/38/14	50.4/45.3/4.3	33	37.5	2	DWR vs Blinding DR**	184/139	[15]
	Chinese	66.2/28.7/5.1	78.9/18/3.1	12.1	19.5	2	DWR vs DR	139/129	[20]
	Chinese	67.7/29/3.2	75/22.6/2.4	17.7	13.7	2	DWR vs DR	279/212	[21]
	Present Study	49.1/45.5/5.4	53.4/35.8/10.8	28.2	28.7	2	DWR vs DR, PDR	110/148	

Table 5: Genotype and Allele frequencies of the targeted intronic polymorphisms in the *VEGF* gene among various published studies

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

The rationale behind diverse outcome could be variations in sample sizes, ethnicity, inclusion criteria of cases and controls, demographic factors and study designs. Apart from rs2146323, the intronic polymorphisms of VEGFA gene did not impose a risk to DR or PDR in the current population. There might be role of mutations/ polymorphisms in VEGFA gene or in other genes like transcription factors such as hypoxia-inducible factor 1-alpha, which regulate VEGF expression and not covered in the study [25].

The regulation of VEGFA protein expression has been shown to be influenced by SNPs in the VEGFA gene, mostly in the promoter and 5' UTR regions suggesting their plausible role in DR pathophysiology [26, 27]. However, the exact mechanism by which the intronic SNPs influence DR susceptibility is obscure. The SNPs in the intron region can be located at exonic enhancers or silencers that regulate transcription and splicing of the VEGFA gene, have not been extensively investigated thus far. The modification in splice sites may affect alternative splicing of VEGF165, a predominating isoform in the eye. This may be the reason for the increased ratio of angiogenic and antiangiogenic isoforms observed during DR [28], but no functional study has been carried out suggesting it. The polymorphisms, rs833069, rs2146323, and rs3025021 are correspondingly located 450 bp 3' to exon 2, 111-bp 5'to exon 3 and 530 bp 5' to exon 7 **[Figure 1]**. At present, various in-silico analyses are predominating to understand the possible role of intronic SNPs in the disease development. ESE Finder, a web-based tool, suggested the presence of C allele at rs833069 increases the potentiality of the branch site and inserts a new splicing site for SRF1 (Serine-rich splicing factor) [29, 30]. On the other hand, rs2146323 and rs3025021 are not located within the predicted splicing factor binding sites, making them little less attractive for the functional analysis. However, the minor allele of rs3025021 showed putative insertion in ESE sites for SRF2 and SRF6. It is perhaps more likely that these SNPs serve to highlight an as-yet-unknown variant. The other possibility for the SNPs in predisposing diabetic patients to DR can be their possible high linkage disequilibrium in particular ethnicity with other SNPs that has functional and /or genetic association with DR.

DR being a complex disease involves multiple genetic and environmental determinants. For the genetic studies of complex human disease, it has been recognized that the interplay among multiple genetic variants is driving disease phenotype rather than single or a few SNPs. The limitation of the study was that other polymorphisms of VEGFA gene and genes other than VEGFA were not taken under consideration that would have influenced the DR susceptibility. Moreover, the current cross-sectional study included comparatively small sample size which may limit the power of the outcome. However, the strengths were including standard diagnosis criteria for the cases by ADA and ETDRS guideline, restricting the study to well-defined Indian ethnicity, and consideration of confounders for the risk assessment of the disease.

[V] CONCLUSION

In summary, our data suggested the potential relationship between the recessive genotype and allele of rs2146323 of VEGFA and PDR. However, other two SNPs did not confer risk to develop DR or PDR. Further, ACC haplotype showed a significant protective effect for PDR among T2D patients. There is a need of studies with larger sample size in the current Indian population that would help in revealing the findings in a better way. Eventually, such candidate genetic studies would assist in identifying patients at high risk of DR comparatively early and can be delayed with more frequent retinal monitoring programs.

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