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Original Research Article

Association of VEGF gene polymorphism with development and progression of diabetic retinopathy in India: a cross-sectional study

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ABSTRACT

Background: Vascular endothelial growth factor (VEGF) polymorphism might be a useful predictive marker for the development and progression of diabetic retinopathy (DR).
Methods: This observational cross-sectional study was done from December 2018 to December 2019. The study included 40 patients of DR and 20 healthy controls. A complete systemic and ocular examination was done including fundus examination, fundus photograph and OCT. The VEGF gene polymorphism of 936C/T, T(-1498)C, G(-1190)A, G(-1154)A and C(-634)G were studied. To study VEGF gene polymorphism the specific primer sequences were used in both forward and reverse directions. Genomic DNA was isolated from a blood sample using a genomic isolation kit and quantification of DNA was done at 260/280 nm on a spectrophotometer. PCR amplification was carried out on each primer.
Results: 936C/T, T(-1498)C, G(-1190)A, G(-1154)A and C(-634)G VEGF gene polymorphism were found in 24 (60%), 34 (85%), 08 (20%), 19 (47.5%) and 21 (52.5%) cases respectively. 936C/T and T(-1498)C gene polymorphism were found in 08 (40%) and 05 (25%) controls respectively. 936C/T and G(-1190)A VEGF gene polymorphism was not statistically significant (p=0.234) (p=0.081) in study groups. T(-1498)C, G(-1154)A and C(-634)G VEGF gene polymorphism were significantly associated with the case and control groups. T(-1498)C, G(-1190)A, G(-1154)A and C(-634)G VEGF gene polymorphism was significantly associated with mild NPDR, moderate NPDR, severe NPDR, PDR and control group (p<.05).
Conclusions: T(-1498)C, G(-1154)A and C(-634)G VEGF gene polymorphism was significantly associated with DR in the population of Western India. T(-1498)C and C(-634)G VEGF gene polymorphism was also associated with the severity of DR.

Keywords: Diabetic retinopathy, Polymorphism, VEGF gene

INTRODUCTION

Diabetic retinopathy (DR) is a chronic progressive, potentially vision-threatening disease of the retinal microvasculature associated with prolonged hyperglycaemia. In 2020, according to the International Diabetes Federation (IDF), 463 million people have diabetes in the world and 88 million people in the Southeast Asia region. Of these 88 million people, 77 million belong to India.[1] The main risk factors associated with rapid onset and progression of retinopathy are the duration of DM, high blood sugar level, hypertension, dyslipidemia etc, but genetic susceptibility is also responsible for variation in incidence and progression of retinopathy. The role of genetics is well explained by the fact that some diabetic patients do not develop retinal complications even after a longer duration of uncontrolled diabetes, while certain individuals develop DR despite tight metabolic control in a short span. Vascular endothelial growth factor (VEGF), a potent angiogenic factor has an important role in diabetic microvascular complications, increased serum and vitreous levels of

VEGF have a strong association with Proliferative DR.[2,3] The VEGF gene is located on chromosome 6p 21.3 region with 7 introns and 8 exons and shows several polymorphisms.[4] The promoter region has a single transcription start site adjacent to a group of Sp1 binding sites.[5] The 3′ UTR (untranslated region) of the VEGF gene is predicted to have mRNA destabilized elements that degrade mRNA stability under normoxic conditions and act synergistically with the 5′UTR and coding region of the gene to bring about mRNA stability during hypoxia.[6]

Our study aimed to establish the disease causative role of VEGF gene polymorphism and its relation with disease progression in DR patients in western India, so we can identify high-risk patients before developing DR.

METHODS

This hospital-based prospective cross-sectional study was done in a tertiary care centre from December 2017 to December 2018. The study included 40 cases of DR and 20 healthy controls. The inclusion criteria were patients with type II DM with DR and who had given informed written consent. The exclusion criteria were previous history of ocular inflammation, ocular trauma and other ocular diseases, type I DM patients, gestational DM, HIV infection and other immunosuppressive conditions and systemic steroids. The study was performed according to the guidelines of the declaration of Helsinki and was approved by an institutional ethical committee. Each patient had undergone a complete ophthalmic examination including fundus examination by direct and indirect ophthalmoscope, slit lamp biomicroscopy with +90D lens, fundus photograph and OCT. DR was classified according to ETDRS classification. The 2 ml blood was drawn in an EDTA vial and sent to multidisciplinary research units for analysis. The disease and its classification were kept blind to a primary scientist who analysed the sample.

Genomic DNA was isolated from a blood sample using a genomic isolation kit and quantification of DNA was done at 260/280 nm on a spectrophotometer. Then quantified DNA was used for further analysis. To study VEGF gene polymorphism in different genotypes the specific primer sequences were used in both forward and reverse directions. The five polymorphisms at the promoter and untranslated (UTR) region of the VEGF gene were studied. The PCR primer of 936C/T, T(1,498)C, G(1,190)A, G(-1,154)A, C(-634)G were taken. Their sequences are shown in Table 1. PCR amplifications were carried out using PCR master mix in 25 µl reaction volumes containing about 25 ng of genomic DNA and 10pmol of each primer (forward and reverse). Amplification was carried out under the following conditions: initial denaturation 94°C for 5 minutes, followed by 30 cycles of denaturation 94°C, annealing for 30 seconds (for each annealing temperature Tm), extension 72°C for 30 seconds, followed by a final extension at 72°C for 2 minutes. Genotyping of each polymorphism was carried out by polymerase chain reaction (PCR).

The continuous data was presented as mean±SD or median and inter-quartile range, as appropriate. Qualitative or categorical variables were described as frequencies and proportions. Proportions were compared using Chi-square or Fischer's exact test whichever was applicable. A p value of <0.05 was considered statistically significant. All calculations were performed using SPSS® version 15 (Statistical Packages for the Social Sciences, Chicago).

RESULTS

The VEGF gene polymorphism 936 C/T at 3′UTR region, T(-1498)C, G(-1190)A and G(-1154)A at promoter region and C(-634)G at 5′ UTR region were studied. In the case (DR) group (n=40) mean age of patients was 61.25±9.64 years, whereas in the control group (n=20) mean age was 60.41±5.3 years. In the case group 23 (57.5%) patients were male while in the control 14 (70%) were male. The urban patients were 16 (60%) and 14 (70%) in the case and control groups respectively. The mean FBS was 180.63±56.52 mg/dl in the case group while in the control group 83.10±14.7 mg/dl. The mean was HbA1c 8.75±3.41% in the case group and 4±6.7% in the control group. In study group 18 patients develop DR within 10 years of DM, 18 patients developed it between 10-20 years and 4 patients developed it after 20 years (Table 2).

Table 1: Primer sequences used to amplify VEGF gene polymorphisms.

Primer Name/ID		Primer sequence 5' --> 3'	No. of bases	Scale (nmol)
936C/T	Forward	5′AAGGAAGAGGAGACTCTGCGCAGAGC-3′	26	25
	Reverse	5′-TAAATGTATGTATGTGGGTGGGTGTGTCTACAGG-3′	24	25
T(-1,498) C	Forward	5′-GTGTGTGCGTGTGGGGTTGGCGG-3′	23	25
	Reverse	5′-CGACCCCCACCAAGGTTCACAG-3′	22	25
G(-1,190)A	Forward	5′-TCCTGCTCCCTCCTCGCCAATG-3′	22	25
	Reverse	5′-TCCACAGTGATTTGGGGAAGTAGA-3′	24	25
G(-1,154)A	Forward	5′-TCCTGCTCCCTCCTCGCCAATG-3′	22	25
	Reverse	5′-GGCGGGGACAGGCGAGCCTC-3′	20	25
C(-634)G	Forward	5′-TTGCTTGCCATTCCCCACTTGA-3′	22	25
	Reverse	5′-CCGAAGCGAGAACAGCCCAGAA-3′	22	25

Hypertension (seven patients) and chronic kidney disease (five patients) were the most common comorbid conditions. In the case group 20.5% (11 patients) had mild NPDR, 35% (14 patients) had moderate NPDR, 25% (10 patients) had severe NPDR and 12.5% (5 patients) had PDR.

Table 2: Clinical characteristics of study population.

Characteristics	Cases (diabetic retinopathy) (n=40)	Healthy control (n=20)
Age (years)	61.25±9.64	60.41±5.28
Sex: male/female	23 / 17	14 / 6
Socioeconomic condition (rural/urban)	16 / 24	6 /14
Fasting blood sugar (mg/dl)	180.63±56.51	83.10±14.70
HbA1c (%)	8.75±3.41	4±6.73
BMI (kg/m^2)	21.55±3.2	18.30±3.37
Duration of disease (years)	12.28±6.05	–
Co-morbid conditions (no. of patients)	16	–

Table 3: VEGF gene polymorphisms positivity in study population.

Gene polymorphism	Case group (n=40) (%)	Control group (n=20) (%)	P value
936C/T	24 (60)	08 (40)	0.234
T(-1,498)C	34 (85)	05 (25)	0.0001
G(-1190)A	08 (20)	00	0.081
G(-1,154)A	19 (47.5)	00	0.000
C(-634)G	21 (52.5)	00	0.000

Table 4: VEGF gene polymorphisms distribution according to severity of diabetic retinopathy.

Type of gene polymorphism	Study group										P value
	Mild NPDR (n=11)		Moderate NPDR (n=14)		Severe NPDR (n=10)		PDR (n=5)		Control (n=20)		
	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve	
936 C/T	08	03	07	07	07	03	02	03	08	12	0.328
T(-1498)C	10	01	14	00	05	05	05	00	05	15	0.001
G(-1190)A	04	07	02	12	01	09	01	04	00	20	0.007
G(-1154)A	04	07	07	07	05	05	03	02	00	20	0.005
C(-634)G	04	07	06	08	07	03	04	01	00	20	0.001

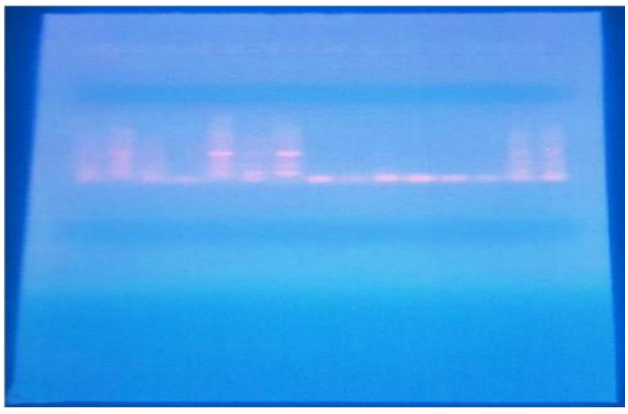

Figure 1: PCR amplification of G(–1190)A VEGF gene polymorphism.

936C/T, T(-1498)C, G(-1190)A, (Figure 1) G(-1154)A and C(-634)G VEGF gene polymorphism were found in 24 (60%), 34 (85%), 08 (20%), 19 (47.5%) and 21 (52.5%) cases respectively. 936C/T and T(-1498)C gene polymorphism were also found in eight (40%) and five (25%) controls respectively. 936 C/T VEGF and G(-1190)A gene polymorphism was statistically not significant in the study population. The p value was 0.234 and 0.08 respectively. T(-1498)C, G(-1154)A, C(-634)G VEGF gene polymorphism was statistically significant with case and control group and p value was 0.0001, 0.000, 0.000 respectively (Table 3) T(-1498)C VEGF gene polymorphism was most commonly (85% cases) associated with the diabetic retinopathy group and G(-1190)A was least (20% cases) associated with diabetic retinopathy.

The 3′ UTR, 936 C/T VEGF gene polymorphism was positive in eight cases of mild NPDR, seven moderate NPDR, seven of severe NPDR and two were of PDR group out of 24 positive cases and eight controls (n=20) respectively. 936 C/T VEGF gene polymorphism was statistically not significant in mild NPDR, moderate

NPDR, severe NPDR, PDR and the control group (p=0.328).

The promoter region T(-1498)C polymorphism was positive in ten cases of mild NPDR, fourteen of moderate NPDR, five of severe NPDR, five of PDR and five control subjects. T(-1498)C VEGF gene polymorphism was statistically significant with mild NPDR, moderate NPDR, severe NPDR, PDR and control group (p= 0.001). Another promoter region polymorphism G(-1190)A was positive in four cases of mild NPDR, two moderate NPDR, one of severe NPDR and one of PDR group out of eight positive cases. None of the control subjects showed G(-1190)A VEGF gene polymorphism. G(-1190)A VEGF gene polymorphism was statistically significant with mild NPDR, moderate NPDR, severe NPDR, PDR and control group (p=0.007). The promoter region G(-1154)A VEGF gene polymorphism were positive in four cases of mild NPDR, seven moderate NPDR, five of severe NPDR and three were of the PDR group out of 19 positive cases. None of the control subjects showed G(-1154)A polymorphism. G(-1154)A polymorphism was statistically significant with mild NPDR, moderate NPDR, severe NPDR, PDR and control group (p=0.005).

The 5′-UTR region C(-634)G polymorphism was positive in four cases of mild NPDR, six of moderate NPDR, seven of severe NPDR and four were in the PDR group, while absent in control subjects. C(-634)G polymorphism was statistically significant with mild NPDR, moderate NPDR, severe NPDR, PDR and control group (p=0.001) (Table 4).

DISCUSSION

DM is a multifactorial disease developed due to the complicated interaction of genetic and environmental factors. In India, 20% of the type 2 diabetes mellitus (DM), the population is estimated to develop DR which suggests that by 2025; nearly 11.5 million adults with diabetes may develop DR. The Wisconsin epidemiologic study of diabetic retinopathy showed that 22.2% of diabetic patients do not develop DR early irrespective of glycemic exposure while 28.8% develop DR early.[7] Understanding the genetic basis of DR would help in identifying high-risk patients and management of this disease. Various genes associated with DR are VEGF, advanced glycation end product (AGE), angiotensin-I converting enzyme, angiotensin II type I receptor, angiotensinogen, plasminogen activator inhibitor-1, $\alpha_2\beta_1$ integrin, peroxisome proliferator-activated receptor gamma, Nitric oxide synthase $(NOS)_3$, Aldose reductase (ALR_2), the receptor for AGEs (RAGE), glucose transporter 1 and transforming growth factor β among others. VEGF is a heparin-binding glycoprotein that is secreted by many cells like endothelial cells, tumour cells, macrophages, platelets, keratinocytes and renal mesangial cells. VEGF, a 45 kD, the homodimeric glycoprotein is secreted from various types of cells in the eye. VEGF is a potent mediator of both vasculogenesis and angiogenesis.[8] VEGF plays a significant role in inducing hyperpermeability of retinal vessels, breakdown of the blood-retinal barrier, and neovascularization.[9,10] VEGF antagonists can reduce retinal vascular permeability and neovascularization, thus inhibiting the development of DR[11-13]; therefore VEGF may be strongly involved in the progression of DR.VEGF gene is highly polymorphic.[11-13] VEGF gene polymorphisms: T(-1,498)C, G(-1,190)A, and G(-1,154)A in the promoter region and C(-634)G and C(-7)T in the 5′-untranslated region (UTR) and C936T and G1612A polymorphisms in the 3′UTR were found to be common in the Japanese population.[14] We have identified five VEGF gene polymorphism in our study showing that all these VEGF gene polymorphisms 936C/T, T(-1498)C, G(-1190)A, G(-1154)A and C(-634)G were present in DR patients in Western India. 936C/T and T(-1498)C VEGF gene polymorphism were also present in healthy controls.

936C/T VEGF gene polymorphism was found in 60% of cases and 40% control. It was not statistically significant (p=0.234) in our study showing that this polymorphism was prevalent in this area. Uthara et al observed in the South Indian cohort that 936 C/T polymorphism in 130 patients of DR and 82 without DR, similar to our study it was not significantly associated with DR and its severity.[15] A study by Awata et al also observed that 936C/T polymorphism in both DR and diabetes without retinopathy group and not statistically significant, similar to our study.[14] Kim et al observed that 936C/T polymorphism was related to DR in Korean population.[16]

T(-1498)C polymorphism was commonly associated with diabetic retinopathy and its severity in our study. It was also present in 25% of healthy control in our study, probably these had more chances of developing DM and DR. It was statistically significant with case and control group (p=0.0001) and was also statistically significant with mild NPDR, moderate NPDR, severe NPDR, PDR and control group (p=0.001) in our study. A study done by Suganthalakshmi et al found T(-1498)C gene polymorphism differed significantly between patients with DR and diabetic without retinopathy group (p=0.0001).[17] When compared to our study it was similar results. A study conducted by Awata et al found T(-1498)C VEGF gene polymorphism was not significant in patients with retinopathy group compared with diabetes without retinopathy.[14]

Our study had shown G(-1190)A VEGF gene polymorphism was least commonly (20%) associated with the diabetic retinopathy group and absent in controls. It was statistically not significant with the case and control group (p=0.081). G(-1190)A polymorphism was statistically significant with mild NPDR, moderate NPDR, severe NPDR, PDR and control group (p=0.007) in our study. Suganthalakshmi B et al found G(-1190)A polymorphism was statistically significant between patients with DR and DWR (diabetic without retinopathy) group (p=0.18).[17] Awata et al found T(-1498)C polymorphism was not statistically significant in patients

with retinopathy group compared with diabetes without retinopathy group.[14]

G(-1154)A VEGF gene polymorphism was positive in 47.5% cases and none of the control subjects showed this polymorphism. G(-1154) A VEGF gene polymorphism was statistically significant with case and control group (p=0.000) in our study. 36.36% (n=11) cases were of mild NPDR, 50% (n=14) moderate NPDR, 50% (n=10) of severe NPDR and 60% (n=5) were of PDR group. This polymorphism was significantly associated with DR and its progression. Awata et al found G(-1154)A VEGF gene polymorphism was non-significant in patients with retinopathy group compared with diabetes without retinopathy.[14]

C(-634)G VEGF gene polymorphism was positive in 52.5% of cases, 36.36% (n=11) cases were of mild NPDR,42.86% (n=14) moderate NPDR, 70% (n=10) of severe NPDR and 80% (n=5) were of PDR group. None of the control subjects showed C(-634)G polymorphism. C(-634)G VEGF gene polymorphism was statistically significant with case and control groups (p=0.000). C(-634)G VEGF gene polymorphism was significantly associated with DR and its progression. A study done by Awata et al observed C(-634)G polymorphism differed significantly between patients without retinopathy and any retinopathy (non-PDR or PDR) (p=0.011).[14] They suggested that the C(-634)G gene polymorphism was primarily associated with diabetic retinopathy. They found that polymorphism might be associated with a severe stage of retinopathy with neovascularisation. Suganthalakshmi et al found statistically significant association of C(-634)G polymorphism with DR (p=0.021) in South Indian cohort.[17] Our study shows similar results when compared to this study. Nakamura et al observed no association of C(-634)G polymorphism in diabetic retinopathy group and control group(no retinopathy).[18]

Limitations of our study were small sample size and cross-sectional study. Serum VEGF level can be included to further clarify the role of VEGF. Studies with large sample sizes should be done to obtain concrete results and longitudinal studies with long-term follow up will be more beneficial. Analysis of gene polymorphism was focused on the specific VEGF gene. Hence, analysis of the multiple genes will give more insight into the disease pathogenesis.

Future outcome of our study

This kind of study will help in identifying potential cases likely to develop retinopathy in the future and help in early diagnosis and treatment. Identifying these pathogenic VEGF gene polymorphisms will enable the design of DNA-based tests that may help physicians to assess their patient's risk for diabetic retinopathy and patient response to therapeutic interventions and will guide their clinical and surgical selection.

CONCLUSION

T(-1498)C, G(-1154)A and C(-634)G VEGF gene polymorphism was significantly associated with DR in the population of Western India. T(-1498)C and C(-634)G VEGF gene polymorphism was also associated with the severity of DR.

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