Original Article

Association of *VEGF* -2549 I/D and *VEGF* +936 C/T Polymorphisms with Chronic Kidney Disease in North-West Indian Patients

Gurleen Kaur Tung, Vasudha Sambyal, Kamlesh Guleria

Human Cytogenetics Laboratory, Department of Human Genetics, Guru Nanak Dev University, Amritsar, Punjab, India

Abstract

Introduction: Chronic kidney disease (CKD) is a complex multifactorial disease in which both genetic and environmental factors influence the onset, development and progression of disease. The genetic variations in the vascular endothelial growth factor (*VEGF*) can influence levels of VEGF protein expression, and thus, susceptibility to progression of kidney diseases. The aim of the present study was to evaluate the association of *VEGF*-2549 I/D and *VEGF* +936 C/T polymorphisms in CKD stage V patients from North-West India. **Methods:** In this case-control study, 166 patients and 166 controls were analyzed. DNA samples were screened for *VEGF* -2549I/D and *VEGF* +936 C/T polymorphisms using polymerase chain reaction–based (PCR) methods. **Results:** The genotype frequency of *VEGF* -2549 I/D was significantly different between patients and controls ($P < 0.05$). ID genotype of *VEGF* -2549 I/D polymorphism was significantly associated with decreased risk of CKD ($P = 0.009$). Genetic model analysis of *VEGF* -2549 I/D polymorphism revealed a significantly decreased risk of CKD in co-dominant ($P = 0.009$), dominant ($P = 0.021$), and over-dominant ($P = 0.012$) models. Genotype and allele frequency of *VEGF* +936 C/T polymorphism was not significantly different between the patient and control groups. Genotype combination analysis revealed that ID-CT genotype combination of *VEGF* -2549 I/D and *VEGF* +936 C/T polymorphisms was associated with decreased CKD risk ($P = 0.047$). **Conclusion:** *VEGF* -2549 ID genotype and ID-CT genotype combination of *VEGF* -2549 I/D and *VEGF* +936 C/T polymorphisms was significantly associated with reduced CKD risk in North-West Indians.

Keywords: *Kidney disease, polymorphism, VEGF*

Introduction

Chronic kidney disease (CKD) is a complex multifactorial disease in which both genetic and environmental factors influence the onset, development, and progression of disease. The progressive and irreversible loss of renal function results in end-stage renal disease (ESRD), necessitating renal replacement therapies (RRTs) for life-sustenance. With a global burden of 13.7%, it is a major risk factor for cardiovascular morbidity and mortality worldwide.[1] Etiological basis of the disease is associated with various pathogenic factors, namely, glomerulonephritis, diabetes, hypertension, and urologic disorders. However, progressive renal microvascular dysfunction, which initiates and promotes interstitial fibrosis, tubular atrophy, and glomerulosclerosis, is a universal pathologic feature of CKD.[2] The microvasculatures of glomerular and peritubular capillaries are critical in kidney disease. Damage to glomerular capillary vasculature leads to proteinuria, and to the peritubular capillary results in chronic hypoxia followed by tubulointerstitium fibrosis.[3,4] Vascular endothelial growth factor (VEGF), a major regulator of blood vessel growth plays a pivotal role in promoting endothelial survival, functional and morphological maintenance of these microvascular networks.[5] In kidneys, VEGF is essential for growth and proliferation of glomerular and peritubular endothelial cells, and thus, maintenance of fenestrae in endothelial cells of glomerular capillaries.[6]

Human *VEGF* or *VEGFA* is located on 6p12 spans 16,272 bp and consists of eight exons.[7] It is reported to be highly polymorphic in the promoter region, 5′ untranslated region (UTR), and 3′ UTR.[8] There are reports on the association of these genetic variations with altered serum and urine VEGF levels[8,9] and diseases including

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Address for correspondence:
Dr. Kamlesh Guleria, Department of Human Genetics, Guru Nanak Dev University, Amritsar - 143005, Punjab, India.
E-mail: guleria_k@yahoo.com

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diabetic nephropathy,[10,11] glomerulonephritis,[12] ESRD,[13] and acute renal allograft rejection.[14-16] *VEGF*-2549 insertion/deletion and *VEGF* +936 C/T have been implicated in a number of diseases with angiogenic basis, and hence are polymorphisms of particular interest.[17-19]

The genetic variations in the *VEGF* can influence levels of VEGF protein expression, and thus susceptibility to progression of kidney diseases. The global burden of CKD is increasing and availability of basic life-sustaining RRTs is limited due to economic constraints.[20] Moreover, screening of *VEGF* polymorphisms can help identify at-risk individuals for graft rejection prior to transplantation.[14,16] Literature regarding the role of *VEGF* -2549 I/D and *VEGF* +936 C/T polymorphisms in various diseases is vast, but is limited to renal complications, especially CKD stage V patients with different etiologies and hemodialysis durations. Therefore, the present study was an attempt to evaluate the association of *VEGF*-2549 I/D and *VEGF* +936 C/T polymorphisms in CKD stage V patients from North-West India.

Methodology

Selection of subjects and collection of genetic material

The present case-control study was carried out after receiving approval from the Institutional Ethics Committee. The physician identified 166 unrelated adult CKD stage V patients (112 males and 54 females) on or starting hemodialysis therapy who were contacted from local hospitals of Amritsar, Punjab. Related, minors, and individuals who were seropositive for hepatitis C or B or HIV or had bacterial infections or any cancer, were not included in this study. Unrelated, healthy, age and gender-matched 166 individuals (107 males and 59 females) from the general population belonging to same geographical area formed the control group. The eGFR values based on the creatinine levels were used to establish the healthy kidney status of the controls. The demographic characteristics, clinical profile and disease history of all the subjects were recorded on a pre-designed structured proforma. After informed written consent, 5 ml venous blood was collected from each subject in EDTA vial.

Analysis of *VEGF*-2549 I/D and *VEGF* +936 C/T polymorphisms

Genomic DNA was extracted from the peripheral blood using standard phenol chloroform method[21] with few modifications. The DNA samples were quantified on 1% agarose gel and screened for *VEGF*-2549I/D and *VEGF* +936 C/T polymorphisms. The *VEGF*-2549I/D promoter polymorphism was screened by direct PCR whereas 3'UTR polymorphism *VEGF* +936 C/T was screened usingpolymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The region of *VEGF* promoter harboring -2549I/D polymorphism was amplified using Forward: 5′-GCTGAGGATGGGGCTGACTAGGTA-3′ and Reverse: 5′GTTTCTGACCTGGCTATTTCCAGG-3′primers. The PCR reaction with a total volume of 10µl, contained 50ng of genomic DNA, 1X *Taq* buffer with 1.5mM $MgCl_2$ (Merck, India), 4 picomoles of each primer (Sigma), 0.4µl of dNTPs mix (Merck, India), and one unit of *Taq* polymerase (Merck, India). For *VEGF* -2549I/D analysis, PCR conditions were the following: initial denaturation at 95°C for 5 minutes followed by 35 cycles with denaturation at 95°C for 45 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 45 seconds, and final extension at 72°C for 10 minutes. The amplified products were analyzed on 2.4% ethidium bromide-stained agarose gel. A band of 229 bp represents I allele whereas band of 211bp represents D allele[Figure 1].

The specific region of *VEGF*containing +936 C/T polymorphism was amplified using Forward: 5′-AGGAAGAGGAGACTCTGCGCAGAGC-3′ and Reverse: 5′-TAAATGTATGTATGTGGG TGGGTGTGTCTACAGG-3′ primers.PCR reaction with a total volume of 15µl, contained 50ng of genomic DNA, 1X *Taq* buffer, 1mM $MgCl_2$ (Merck, India), 6 picomoles of each primer (Sigma), 0.2µl dNTPs mix (Merck, India) and one unit of *Taq* polymerase (Merck, India). The amplification conditions used were: initial denaturation at 95°C for 5 minutes, followed with a denaturation at 95°C for 45 seconds, annealing at 59°C for 30 seconds and an extension at 72°C for 45 seconds for 35 cycles with final extension at 72°C for 10 minutes. The amplified PCR products of 217bp were digested with *Nla*III restriction enzyme (New England Biolabs, Beverly, MA) at 37°C overnight. Restriction digestion reaction products were analyzed on 2.4% ethidium bromide–stained agarose gel. Two fragments of 122bp and 85bp indicates +936T allele, whereas the undigested fragment of 207bp

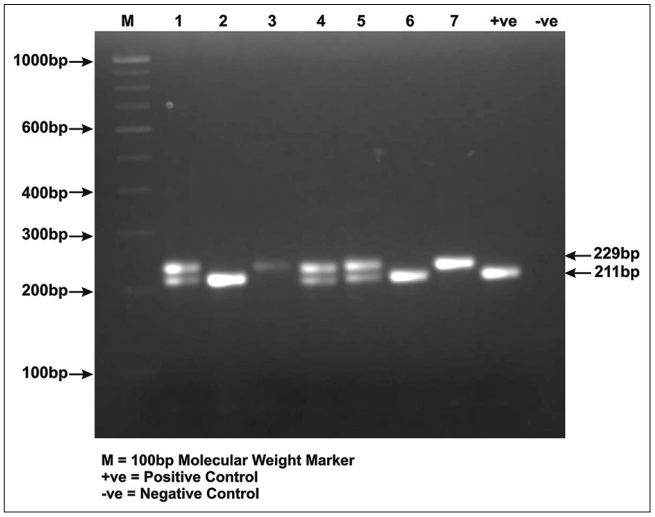

Figure 1: A photograph of 2.4% ethidium bromide –stained agarose gel demonstrating *VEGF* -2549I/D polymorphism. Lane 1, 4, 5 represents ID genotype, Lane 2, 6, +ve control represents DD genotype and Lane 3, 7 represents II genotype

represents +936C allele [Figure 2].The details of reaction composition and conditions used have been presented in our published study.[22] To ensure genotyping accuracy, positive and negative controls were used in every batch of reaction. The PCR assay-based results were validated by reanalyzing the 10% of randomly selected samples.

Statistical analyses

The data on variables are expressed as number or as percentage, and as mean ± standard deviation. Differences between the patient and control groups were analyzed by independent Student's *t* test while multiple comparisons were carried out using one-way ANOVA. The allele frequencies were tested for the Hardy–Weinberg equilibrium (HWE) for both patients and controls using the Chi-square test. This test was also used to evaluate the differences in the *VEGF* genotype and allele frequencies between the patient and control groups, and also as a function of various general, demographic, and clinical parameters. Odds ratio (OR) and its 95% confidence interval (CI) were used to assess the association between genotypes and alleles with the disease risk. Haplotypes were constructed using SNPStats.[23] Statistical significance level was set at $P < 0.05$.

Results

General demographic and clinical profile of subjects

The patient group (n = 166) comprised of unrelated patients in CKD stage V in the age range of 18–80 years (mean = 50.87 ± 13.36 years) with high frequency of males (67.47%). Mean age of unrelated healthy individuals was 48.24 ± 12.23 years [Table 1]. About 74.69% of the patients were on hemodialysis therapy for 8 months to 6 years. Ongoing hemodialysis therapy comprised of groups on once-a-week (23.49%), twice-a-week (25.30%), thrice-a-week (12.05%), and fortnightly (15.66%) regimens with 25.30% yet to initiate hemodialysis. Serum creatinine (7.37 ± 3.37 mg/dl) and urea (125.46 ± 34.90 mg/dl) levels were elevated in the patients. Patients were on prescribed medications from past 9.24 ± 0.70 years.

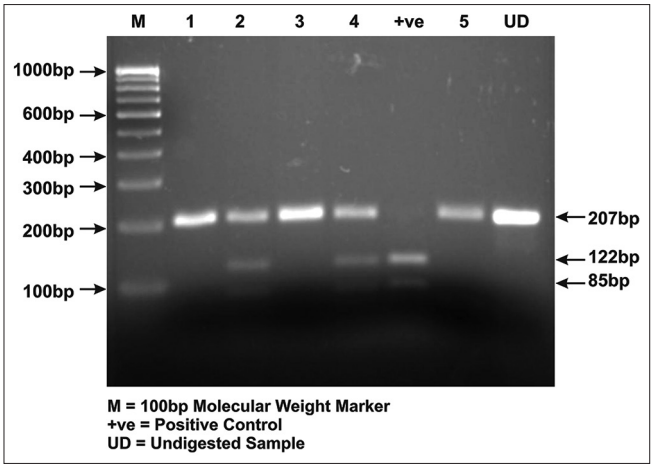

Figure 2: A photograph of 2.4% ethidium bromide–stained agarose gel demonstrating restriction digestion pattern of *VEGF* +936C/T polymorphism. Lane 1, 3, 5 represents CC genotype, Lane 2, 4 represents CT genotype and +ve control represents TT genotype

Table 1: General Demographic and Clinical variables of the Study Group

Characteristics	Categories/ Types	Patients *n* (%)	Controls *n* (%)
Age (years)	≤50	80 (48.19)	94 (56.63)
	>50	86 (51.80)	72 (43.37)
	Mean	50.87±13.36	48.24±12.24
Gender	Male	112 (67.47)	107 (64.46)
	Female	54 (32.53)	59 (35.54)
Socioeconomic status[24]	Upper	10 (6.02)	04 (2.41)
	Upper Middle	52 (31.33)	52 (31.33)
	Lower Middle	46 (27.71)	54 (32.53)
	Upper Lower	58 (34.94)	56 (33.73)
Diet	Vegetarian	119 (71.69)	126 (75.90)
	Non-vegetarian	47 (28.31)	40 (24.10)
Smoking habit	Yes	05 (3.01)	07 (4.22)
	No	161 (96.99)	159 (95.78)
Alcohol consumption	Yes	12 (7.23)	35 (21.08)
	No	118 (71.08)	125 (75.30)
	Ex-drinker	36 (21.67)	06 (3.61)
Mobile usage	Yes	129 (77.71)	137 (82.53)
	No	37 (22.29)	29 (17.47)
General obesity[25] BMI (kg/m²)	Underweight	13 (7.83)	04 (2.41)
	Normal	95 (57.23)	76 (45.78)
	Overweight	30 (18.07)	26 (15.66)
	Obese	28 (16.87)	60 (36.14)
	Mean	22.09±3.20	24.28±4.12
Blood Pressure	SBP (mmHg)	137.49±16.77	131.59±14.14
	DBP (mmHg)	85.72±8.05	84.78±10.87
	PP	51.77±12.59	46.81±10.33
	MAP	102.98±10.09	100.39±11.03
Creatinine levels[ǂ] (mg/dl)		7.37±3.37	1.12±0.61
Urea levels (mg/dl)[#]		125.46±31.78	-
Comorbidity	Diabetes	51 (30.72)	-
	Hypertension	37 (22.29)	-
	Diabetes + Hypertension	13 (7.83)	-
	Unknown	65 (39.16)	-
Dialysis Duration (Years)	≤1	57 (34.34)	-
	>1	67 (40.36)	-
	Non-Dialyzed	42 (25.30)	-

BMI- Body Mass Index; CKD-EPI- Chronic kidney disease epidemiology equation; DBP- Diastolic Blood Pressure; eGFR- estimated glomerular filtration rate; MAP- Mean Arterial Pressure; SBP-Systolic Blood Pressure; PP- Pulse Pressure. [ǂ]Normal range 0.80-1.40 mg/dl for males and 0.60-1.40 mg/dl for females. [#]Normal range 8-20 mg/dl; Data not available for controls. [$]Calculated using variables of age, gender, and serum creatinine levels (http://www.nkdep.nih.gov/professionals/gfr_calculators/index.htm)

VEGF-2549 I/D and *VEGF* +936 C/T polymorphisms and disease risk

Genotype and allele frequencies of *VEGF*-2549 I/D and *VEGF* +936 C/T polymorphisms in patient and control groups are detailed in Table 2. The genotype distributions of *VEGF* -2549 I/D and *VEGF* +936 C/T polymorphisms were in HWE ($P > 0.05$; except *VEGF* -2549 I/D in controls). The frequency of DD (33.73 vs 22.29%), ID (46.99 vs 60.84%) and II (19.28 vs 16.87%) genotypes of *VEGF* -2549 I/D was significantly different between patients and controls ($P < 0.05$). ID genotype of *VEGF* -2549 I/D polymorphism was significantly associated with decreased risk of CKD ($P = 0.009$). The frequency of CC (82.53% vs 83.13%) and CT (16.27% vs 16.87%) genotypes of *VEGF* +936 C/T polymorphism was not significantly different between the patient and control groups. The variant genotype TT was only observed in the patient group (1.20%). The frequency of T allele was slightly higher in patients (9.34%) compared to controls (8.43%).

The genetic model analysis of *VEGF* -2549 I/D polymorphism revealed a significant decreased risk of CKD in co-dominant (OR: 0.510, 95% CI: 0.307–0.849; $P = 0.009$), dominant (OR: 0.563, 95% CI: 0.346-0.917; $P = 0.021$) and over-dominant (OR: 0.570, 95% CI: 0.369–0.882; $P = 0.012$) models [Table 3]. However, none of the genetic models of *VEGF* +936 C/T polymorphism showed any significant disease association.

The frequency of distribution of *VEGF* -2549 I/D and *VEGF* +936 C/T polymorphisms as a function of general demographic (age, gender) and disease-specific variables showed no association with the disease (data not shown). Genotype combination analysis [Table 4] showed that ID genotype of *VEGF* -2549 I/D and CT genotype of *VEGF* +936 C/T polymorphisms was associated with decreased CKD risk (OR: 0.450, $P = 0.047$). Haplotypes constructed for *VEGF*-2549 I/D and *VEGF* +936 C/T polymorphisms did not reveal any significant disease-risk association [Table 4]. Analysis of association of *VEGF* -2549 I/D and *VEGF* +936 C/T polymorphism with creatinine level showed non-significant results (data not shown).

Discussion

VEGF is essential for maintenance of glomerular filtration barrier and its dysregulation has been reported to be associated with various glomerular and associated diseases.[26] In the present case-control study, *VEGF* -2549 I/D promoter and *VEGF* +936 C/T 3′UTR polymorphism were screened in 166 CKD patients and 166 controls. We observed that ID genotype of *VEGF* -2549 I/D polymorphism was significantly associated with decreased risk of CKD. In the literature, it has been reported that *VEGF* promoter and 3′UTR polymorphism are associated with varying VEGF production. It has also been documented that D allele of *VEGF* -2549 I/D polymorphism was associated with 1.95-fold increased transcriptional activity as compared to I allele.[27] DD genotype and D allele of *VEGF* -2549 I/D polymorphism were associated with susceptibility

Table 2: Genotype and allele frequency distributions of *VEGF* -2549 I/D and *VEGF* +936 C/T polymorphisms in patients and controls

Variant	Patients *n* (%)	Controls *n* (%)	χ^2 (*P*)	OR (95%CI)	*P*
-2549 I/D (rs35569394)					
Genotype					
DD	56 (33.73)	37 (22.29)	7.104 (**0.029**)	Reference	
ID	78 (46.99)	101 (60.84)		0.510 (0.307-0.849)	**0.009**
II	32 (19.28)	28 (16.87)		0.775 (0.392-1.454)	0.401
Allele					
D	190 (57.23)	175 (52.71)	1.193 (0.275)	Reference	
I	142 (42.77)	157 (47.29)		0.833 (0.613-1.131)	0.242
Hardy-Weinberg Equilibrium	χ^2=0.268	χ^2=8.068			
	P=0.605	*P*=**0.005**			
+936 C/T (rs3025039)					
Genotype					
C/C	137 (82.53)	138 (83.13)	2.022 (0.364)	Reference	
C/T	27 (16.27)	28 (16.87)		0.971 (0.544-1.733)	0.926
T/T	02 (1.20)	-		-	
Allele					
C	301 (90.66)	304 (91.57)	0.074 (0.785)	Reference	
T	31 (9.34)	28 (8.43)		1.118 (0.655-1.909)	0.683
Hardy-Weinberg Equilibrium	χ^2=0.257	χ^2=1.408			
	P=0.612	*P*=0.235			

Values in bold are significant; OR: Odds Ratio, CI: Confidence Interval

Table 3: Genetic models analyses of *VEGF* polymorphisms

Variant	Model	Genotypes	Patients *n* (%)	Controls *n* (%)	OR (95% CI)	*P*
-2549 I/D (rs35569394)	Co dominant	DD	56 (33.73)	37 (22.29)	Reference	
		ID	78 (46.99)	101 (60.84)	0.510 (0.307-0.849)	**0.009**
		II	32 (19.28)	28 (16.87)	0.775 (0.392-1.454)	0.401
	Dominant	DD	56 (33.73)	37 (22.29)	Reference	
		ID + II	110 (66.27)	129 (77.71)	0.563 (0.346-0.917)	**0.021**
	Recessive	DD+ID	134 (80.72)	138 (83.13)	Reference	
		II	32 (19.28)	28 (16.87)	1.177 (0.672-2.061)	0.569
	Over dominant	DD + II	88 (53.01)	65 (39.16)	Reference	
		ID	78 (46.99)	101 (60.84)	0.570 (0.369-0.882)	**0.012**
+936 C/T (rs3025039)	Co dominant	CC	137 (82.53)	138 (83.13)	Reference	
		CT	27 (16.27)	28 (16.87)	0.971 (0.544-1.733)	0.926
		TT	02 (1.20)	-		
	Dominant	CC	137 (82.53)	138 (83.13)	Reference	
		CT + TT	29 (17.47)	28 (16.87)	1.043 (0.589-1.846)	0.884
	Recessive	CC + CT	164 (98.80)	166 (100)	Reference	
		TT	02 (1.20)	-		NC
	Over dominant	CC + TT	139 (83.73)	138 (83.13)	Reference	
		CT	29 (17.47)	28 (16.87)	1.028 (0.581-1.819)	0.924

Values in bold are significant; OR: Odds Ratio, CI: Confidence Interval; NC: Not calculated

Table 4: Combined Genotype and Haplotype Analysis of *VEGF* -2549 I/D and *VEGF*+936 C/T polymorphisms in Patients and Controls

	Patient *n*(%)	Control *n*(%)	OR (95% CI)	*P*
Genotype Combinations[#]				
DD-CC	50 (30.12)	37 (22.29)	Reference	
ID-CC	63 (37.95)	78 (46.99)	0.598 (0.349-1.025)	0.061
II-CC	24 (14.46)	23 (13.86)	0.772 (0.379-1.575)	0.477
DD-CT	06 (3.61)	-	NC	
ID-CT	14 (8.43)	23 (13.86)	0.450 (0.205-0.991)	**0.048**
II-CT	07 (4.22)	05 (3.01)	1.036 (0.305-3.523)	0.955
Haplotypes[#]				
D-C	0.540	0.527	Reference	
I-C	0.367	0.389	0.925 (0.669-1.278)	0.635
I-T	0.061	0.084	0.733 (0.401-1.340)	0.313
D-T	0.032	0	NC	

Values in bold are significant; [#]*VEGF* -2549 I/D and *VEGF* +936 C/T; OR: Odds Ratio, CI: Confidence Interval. NC: Not calculated

to diabetic nephropathy in British Caucasoid patients.[25] DD genotype and D allele was associated with increased risk of hypertensive nephrosclerosis in North Indians.[13] Significant association of D allele with increased risk to diabetic nephropathy has been reported in North Indian population.[11]

A comparison of genotype frequencies of the present study with those reported in literature in kidney disease revealed that genotype frequencies of *VEGF* -2549 I/D polymorphism observed in the present study are similar as reported in ESRD patients from North India.[13]The genotype frequencies of *VEGF* +936 C/T polymorphism observed in the presented study were comparable with Caucasian kidney graft patients.[14] ID-CT genotype combination of *VEGF* -2549 I/D and *VEGF* +936 C/T polymorphisms was associated with decreased CKD risk in the present study. It has been reported that carriers of the T allele of *VEGF* +936 C/T polymorphism have significantly lower VEGF plasma levels as compared to non-carriers.[9] CT and TT genotype and T allele of *VEGF* +936 C/T polymorphism was associated with increased risk of kidney disease patients with different etiologies like chronic glomerulonephritis, chronic interstitial nephritis, hypertensive nephrosclerosis in North Indians.[13]

As a function of disease-etiologies (diabetic nephropathy, hypertensive nephropathy, miscellaneous), non-significant differences in frequency distribution of the studied polymorphisms were observed in the present study. Similar to our findings, no association of *VEGF* +936 C/T polymorphism with glomerulonephritis was observed in Turkish population.[12] Stratification of the patients on the basis of demographic and clinical variables revealed non-significant differences. Though there was a preponderance of male patients in the present study, non-significant association of *VEGF* -2549 I/D and *VEGF* +936 C/T polymorphisms was observed in males. In Japanese patients it has been demonstrated that CC genotype of *VEGF* +936 C/T polymorphism was not only associated with risk to ESRD but also associated with increased VEGF levels and mRNA stability in males.[28]The *VEGF* +936 CT genotype and T allele have been reported to be associated with good outcome in renal transplantation.[14] T allele was also found to be associated with acute renal allograft rejection.[29] Significant

association of *VEGF*-2549 DD genotype with graft failure and protective association of *VEGF* +936 CC genotype in kidney allograft recipients was reported in North Indians.[16] The intrarenal VEGF therapy at both, preventive and interventional stages has been reported to be associated with improved renal microvasculature and function and reduction in renal fibrosis.[30] Therefore, screening of the *VEGF* polymorphisms holds relevance as it is an important angiogenic factor implicated in renal pathologies.

Conclusion

Present case-control study revealed that *VEGF* -2549 ID genotype and ID-CT genotype combination of *VEGF* -2549 I/D and *VEGF* +936 C/T polymorphisms was significantly associated with decreased CKD risk in North-West Indians.

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Conflicts of interest

There are no conflicts of interest.

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