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CLINICAL RESEARCH

Vascular Endothelial Growth Factor Gene Polymorphism Is Associated With Long-term Kidney Allograft Outcomes

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Introduction: Vascular endothelial growth factor (VEGF) regulates vasculogenesis in physiological and pathological states. We evaluated the role of VEGF single-nucleotide polymorphisms (SNPs) −1154 G/A, −2578 C/A, +936 C/T, and −2549 Ins/Del in chronic allograft nephropathy.

Methods: Blood samples were collected before renal transplantation, and DNA was extracted. Genotyping of VEGF SNPs −1154 G/A (rs1570360), −2578 C/A (rs699947), +936 C/T (rs112005313), and −2549 Ins/Del (18bpindel) polymorphisms were carried out. Relative quantification of VEGF-A mRNA expression for 4 VEGF SNPs were quantified by the $2^{-\Delta\Delta Ct}$ algorithm. Kidney allografts were categorized into graft loss (n = 98) and normally functioning (n = 174) groups. Genotype frequencies were calculated using additive, dominant, and recessive models. Hardy–Weinberg Equilibrium was assessed between outcome groups by standard procedure using χ^2 analysis. The cumulative allograft survival was estimated by Kaplan–Meier analysis and compared among VEGF genotypes by the log-rank test. Study limitations were the lack of VEGF serum levels, donor-specific antigens, and protocol biopsies.

Results: There was an association of AA (hazard ratio = 2.42, $P = 0.0001$) and CA (hazard ratio = 1.83, $P = 0.009$) genotypes of −2578 C/A SNP with graft loss. After adjustment for transplant-related covariates, associations of VEGF SNPs −2578 C/A and −2549 Ins/Del with graft failure were found to be significant. There was prolonged graft survival for cases with the CC genotype of VEGF −2578 C/A SNP. The carrier −2578*CC, −1154*GG, and +936*CC genotypes were shown to have a strongly protective association. There was no association with posttransplantation lymphomas.

Conclusion: Recipients of kidney allografts possessing low-producing VEGF genotypes are associated with less prolonged graft survival.

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The management of chronic allograft dysfunction (CAD) continues to be a challenge. The cause of CAD is multi-factorial, and may include a combination of immunological and nonimmunological factors.[1] Renal microvascular injury has been a prominent feature, possibly due to a decrease in pro-angiogenic survival factors.[2]

Vascular endothelial growth factor (VEGF) is a central regulator of vasculogenesis both in physiological and in pathological states. It induces endothelial fenestration and maintains vascular permeability.[3–5] VEGF also supports vascular survival by preventing endothelial apoptosis. VEGF has been shown to repair the interstitial tubule compartment in cyclosporine nephrotoxicity, whereas VEGF mRNA level has been up-regulated in tubules in hypoxic states.[6,7] Although VEGF is expressed constitutively, its function in pathological states is less clearly defined. VEGF mRNA and protein are increased in pathological conditions associated with a macrophage inflammatory infiltrate.

Previous studies have shown an association of VEGF-A polymorphism with end-stage renal disease[8]

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and early acute rejection.[9] Our hypothesis is that VEGF may contribute to the pathogenesis of CAD. Herein, we report the results of a prospective, single-center study seeking an association of 4 functionally relevant VEGF SNPs (VEGF −1154 G/A, −2578 C/A, +936 C/T, and −2549 Ins/Del) with long-term kidney allograft outcomes.

METHODS

Patient Selection and Follow-up

A total of 272 renal allograft recipients who underwent renal transplantation at our institution between 2005 and 2011 were included in the study. All of the patients were followed up after renal transplantation until the end of the study period (December 2015). Clinical events of graft dysfunction, allograft biopsy for cause, infections, hospitalization, graft failure, initiation of dialysis therapy, malignancies, and death were recorded prospectively. Renal allograft biopsy was performed for an unexplained acute rise of serum creatinine by $>$ 25% of baseline, a progressive rise of creatinine, new-onset or increase in pre-existing proteinuria, CAD without an apparent cause, or whenever a specific diagnosis was considered. The updated Banff 2007 nomenclature was used to score histologic findings and to classify diagnostic categories.[10] Graft failure was defined as estimated glomerular filtration rate (eGFR, calculated from the 4-variable Modification of Diet in Renal Disease [MDRD] Study equation) of $<$ 15 ml/min per 1.73 m^2 or initiation of dialysis. The study was approved by the institutional ethics committee of the Sanjay Gandhi Postgraduate Institute of Medical Sciences and Department of Biotechnology, Government of India, New Delhi, India. Informed consent was obtained from all individuals, and the study was performed according to the principles of the Declaration of Helsinki.

DNA Extraction and VEGF Genotyping

Blood samples were collected before renal transplantation, and DNA was extracted using a QIAmp DNA Blood Mini Kit (Brand GMbH and Co KG, Cat. No. 51104; Qiagen, Valencia, CA). Genotyping of VEGF SNPs −1154 G/A (rs1570360), −2578 C/A (rs699947), +936 C/T (rs112005313), and −2549 Ins/Del (18bp indel) polymorphisms were carried out as previously reported.[8]

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was isolated from whole blood using Trireagent (Invitrogen, Carlsbad, CA). Complementary DNA (cDNA) was prepared from 5 μg of total RNA using oligo (dT) primers and Moloney murine leukemia virus reverse transcriptase (Agilent Technologies, Santa Clara, CA). The real-time reaction was performed at 42°C for 50 minutes. Real-time polymerase chain reaction amplification was carried out using 4 VEGF-A SNP-specific primers. In addition, real-time polymerase chain reaction amplification of GAPDH (endogenous control) was carried out using primers to estimate the amount of RNA in all samples. Relative quantification of VEGF-A mRNA expression for 4 VEGF SNPs were quantified by the $2^{-\Delta\Delta Ct}$ algorithm with GAPDH as the housekeeping gene and a commercial human cDNA as the universal reference.

Statistical Analysis

Data were expressed as percentages or as mean ± SD and compared using the χ^2 test or analysis of variance as appropriate. Genotype frequencies were calculated using additive, dominant, and recessive models. Hardy−Weinberg equilibrium was assessed between outcome groups by standard procedure using χ^2 analysis. Variables included patient age, patient gender, diabetic status, donor age, donor gender, donor glomerular filtration rate (GFR), number of human leukocyte antigen (HLA) mismatches, and acute rejection episodes. These were adjusted when significance of VEGF SNPs with graft loss was found. Graft loss (GFR $<$ 15 ml/min per 1.73 m^2 or initiation on dialysis) was considered as an event. Cumulative allograft survival was estimated by Kaplan−Meier analysis and compared among VEGF genotypes by the log-rank test. Unadjusted and adjusted hazard ratios for graft loss were calculated for various genotype models by using Cox univariate and multivariate regression, respectively. For all analyses, a value of $P \leq 0.05$ was considered significant. All the analyses were performed with SPSS 16.0 software (SPSS Inc, Chicago, IL).

RESULTS

Recipient Characteristics and Outcomes

Baseline demographic and clinical characteristics were shown in Table 1. The mean duration of follow up was 48.1 ± 28.7 months (range, 3−126 months). During the follow-up period, 98 grafts (36%) were lost. Histologic diagnosis was available for 86 patients. Causes of graft failure were acute rejection (n = 10), vascular causes (n = 3), and surgical complications (n = 6), chronic antibody-mediated rejection including transplant glomerulopathy (n = 25), chronic calcineurin toxicity (n = 14), recurrence of primary disease (n = 8), *de novo* glomerulonephritis (n = 8), BK polyoma virus nephropathy (n = 3), and chronic pyelonephritis (n = 2). Nonspecific interstitial fibrosis and tubular atrophy (IF/TA) was found in 19 renal biopsy samples. Of the 98 patients with graft loss, 64 patients died (61 due to cardiovascular events and 3 due to infection), and 174

Table 1. Demographic and clinical characteristics of the study population

Characteristics	Mean ± SD, or n	Min−Max, or (%)
Recipient age (yr)	38.2 ± 11.6	16–65
Recipient gender		
Male	227	83.5%
Female	45	16.5%
Recipient BMI (kg/m^2)	23.1 ± 1.6	17.3–30.4
Basic disease		
CGN	122	44.8%
CIN	92	33.8%
DKD	31	11.4%
ADPKD	3	1.1%
Other	24	8.8%
Diabetes	33	12.1%
Donor age (yr)	44.4 ± 10.9	21–76
Donor gender		
Male	71	26.1%
Female	201	73.9%
Donor GFR (ml/min)	39.2 ± 6.8	27–68
HLA mismatch	3.0 ± 1.1	1–5
HLA-A mismatch	146	53.7%
HLA-B mismatch	176	64.7%
HLA-DR mismatch	148	54.4%
Delayed graft function	14	5.1%
Immunosuppression		
Prednisolone	267	98.2%
Cyclosporine	113	41.5%
Tacrolimus	155	57.0%
Mycophenolate	255	93.8%
Acute rejection		
Early (≤ 3 mo)	76	27.9%
Late (> 3 mo)	54	19.9%
Cellular	48	17.6%
Antibody-mediated	40	14.7%
Mixed	42	15.4%
Follow-up duration (mo)	48.1 ± 28.7	3–126
Graft loss	98	36.0%
Death	64	23.5%

ADPKD, autosomal dominant polycystic kidney disease; BMI, body mass index; CGN, chronic glomerulonephritis; CIN, chronic interstitial nephritis; DKD, diabetic kidney disease; GFR, glomerular filtration rate; HLA, human leukocyte antigen.

patients had functioning grafts during the study period. There was 1 case of lymphoma (confined to the allograft) in the entire cohort.

Distribution of VEGF Variants

Distribution of VEGF SNPs −1154 G/A, −2578 C/A, +936 C/T, and −2549 Ins/Del were similar among male and female participants and were in Hardy−Weinberg equilibrium among the 2 categories of renal allograft outcomes: graft loss, and patient survival with a functioning graft. All the studied SNPs showed minor allele frequencies > 2%.

Expression Pattern for VEGF-A−Specific SNPs

We carried out the mRNA expression for 4 VEGF-A SNPs. Comparisons were drawn between cases with CAD versus functioning graft (Figure 1). The VEGF-A

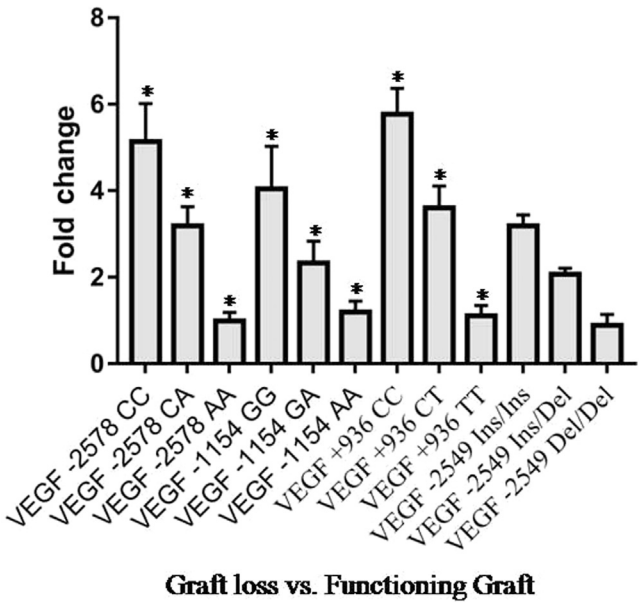

Figure 1. mRNA expression pattern for individual vascular endothelial growth factor (VEGF) single-nucleotide polymorphisms.

mRNA fold change was represented as mean ± SD. Increased mRNA levels were observed for patients with wild-type genotypes such as −1154 GG (4.04 ± 0.99, $P = 0.002$), −2578 CC (5.14 ± 0.88, P value = 0.036), and +936 CC (5.77 ± 0.60, $P = 0.031$). Decreased mRNA levels were observed for patients with mutant genotypes such as −1154 AA (1.20 ± 0.25, $P = 0.041$), −2578 AA (0.99 ± 0.20, $P = 0.006$), and +936 TT (1.11 ± 0.24, $P = 0.020$). In comparison to wild-type genotypes, heterozygous genotypes such as VEGF −2578 CA (3.19 ± 0.44, $P = 0.002$), VEGF −1154 GA (2.33 ± 0.51, $P = 0.041$), and VEGF +936 CT (3.60 ± 0.51, $P = 0.035$) showed decreased mRNA level. No significance was found for any of the genotypes with VEGF −2549 Ins/Del SNP.

Graft Survival

Cumulative graft survival in relation to the 4 VEGF polymorphisms was estimated using Kaplan−Meier analysis (Figure 2). Only the SNP at −2578 C/A was significantly associated with graft survival (log rank $P = 0.002$). The 1-, 3-, 5-, and 10-year graft survival rates were better for patients with the CC genotype (96.7%, 93.4%, 80.3%, and 56.2%) and CA genotype (86.6%, 76.8%, 66.5%, and 40.2%) as compared to the AA genotype (83.6%, 67.1%, 45.5%, and 32.7%).

Regression Analysis

Both unadjusted and adjusted hazard ratios for graft loss were calculated using different models for 4 SNPs (Table 2). Transplant-related donor and recipient characteristics were compared among the 3 genotypes of VEGF SNPs −2578 C/A and −2549 Ins/Del (Table 3).

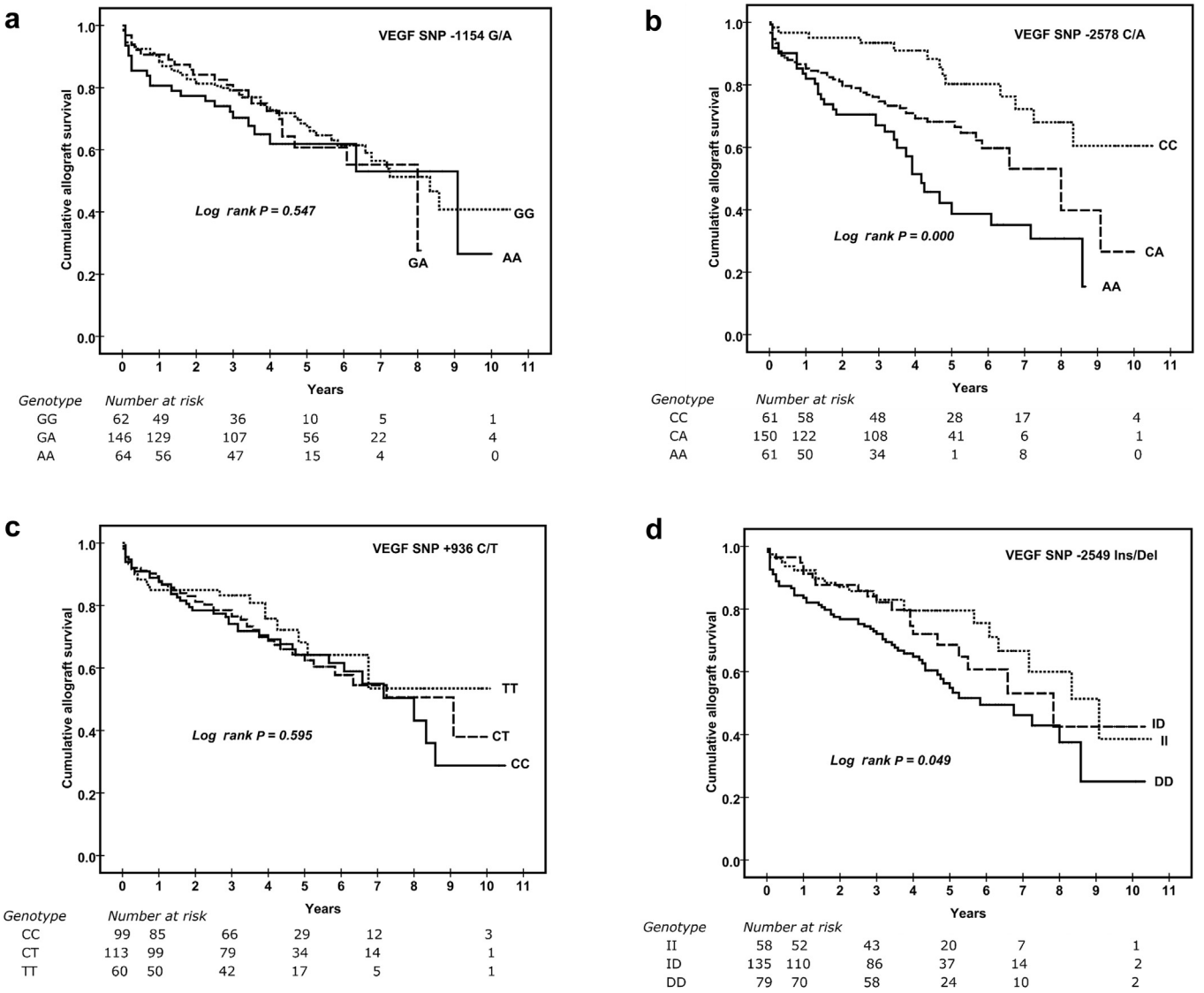

Figure 2. Association of vascular endothelial growth factor (VEGF) single-nucleotide polymorphisms (SNPs) with long-term graft survival. Kaplan–Meier survival plots generated for kidney allograft survival for wild homozygous, heterozygous, and mutant homozygous genotypes of (a) VEGF -1154 G/A, (b) VEGF -2578 C/A, (c) VEGF +936 C/T, and (d) VEGF -2549 Ins/Del SNPs.

Even after adjustment for transplant-related covariates, the association of VEGF SNPs −2578 C/A and −2549 Ins/Del with graft failure was significant. For SNP −2578 C/A, the presence of AA genotype (vs. CC genotype, adjusted HR = 2.42, 95% CI = 1.79−4.52, $P = 0.000$) or CA genotype (vs. CC genotype, adjusted HR = 1.83, 95% CI = 1.16−3.07, $P = 0.009$) were independent predictors for graft loss. The wild-type genotype (dominant model) and C carrier genotype (recessive model) of SNP −2578 C/A had a protective association against risk of graft loss compared to the mutant and non−C carrier genotype, respectively. For SNP −2549 Ins/Del, the presence of the Del/Del genotype (vs. Ins/Ins genotype, adjusted HR = 2.35, 95% CI = 1.25−4.39, $P = 0.007$) was associated with increased risk of graft loss; however, associations were not significant in either the dominant or recessive model.

DISCUSSION

Long-term outcome of the graft depends on adaptive response of renal tissue against a series of time-dependent immunologic and nonimmunologic injuries after transplantation.[11] Chronic antibody-mediated rejection and calcineurin inhibitor toxicity are the most common causes of renal allograft failure. However, in many subjects, no specific cause can be identified, and it is classified as nonspecific IF/TA. VEGF is a central regulator of angiogenesis, both in physiological and in pathological states. Our single-center prospective study showed that there was a significant association of AA

Table 2. Genotype frequency and hazard ratios for graft loss for 4 VEGF SNP genotypes

	Graft loss n = 98	Functioning graft n = 174	Unadjusted			Adjusted[a]		
			HR	95% CI	P	HR	95% CI	P
VEGF −1154 G/A								
AA (additive model)	22 (22.4)	42 (24.1)	1.08	0.58–2.01	0.789	1.05	0.55–2.02	0.869
GA (additive model)	53 (54.1)	93 (53.4)	1.12	0.54–2.33	0.75	1.10	0.50–2.41	0.810
GG	23 (23.5)	39 (22.4)		Reference			Reference	
GG versus GA + AA (dominant model)			1.06	0.59–1.91	0.842	0.94	0.50–1.77	0.859
GG + GA versus AA (recessive model)			1.09	0.61–1.97	0.753	0.93	0.50–1.74	0.835
VEGF −2578 C/A								
AA (additive model)	33 (33.7)	28 (16.1)	3.10	1.68–5.73	0.000[a]	2.42	1.79–4.52	0.000[a]
CA (additive model)	52 (53.1)	98 (56.3)	1.95	1.28–2.94	0.004[a]	1.83	1.16–3.07	0.009[a]
CC	13 (13.3)	48 (27.6)		Reference			Reference	
CC versus CA + AA (dominant model)			0.40	0.20–0.78	0.008[b]	0.38	0.19–0.77	0.008[b]
CC + CA versus AA (recessive model)			0.37	0.21–0.67	0.001[b]	0.33	0.18–0.62	0.001[b]
VEGF +936 C/T								
TT (additive model)	17 (17.3)	43 (24.7)	1.49	0.75–2.95	0.245	1.40	0.69–2.83	0.347
CT (additive model)	42 (42.9)	71 (40.8)	1.64	0.82–3.28	0.159	1.61	0.78–3.30	0.193
CC	39 (39.8)	60 (34.5)		Reference			Reference	
CC versus CT + TT (dominant model)			1.25	0.75–2.09	0.382	1.28	0.75–2.19	0.356
CC + CT versus TT (recessive model)			1.56	0.83–2.92	0.162	1.49	0.78–2.86	0.224
VEGF −2549 Ins/Del								
DD (additive model)	21 (21.4)	58 (33.3)	2.34	1.28–4.28	0.006[a]	2.35	1.25–4.39	0.007[a]
ID (additive model)	62 (63.3)	73 (42.0)	0.96	0.44–2.08	0.925	1.04	0.46–2.30	0.922
II	15 (15.3)	43 (24.7)		Reference			Reference	
DD versus ID + II (dominant model)			1.83	1.03–3.26	0.039[a]	1.81	1.03–3.42	0.065
DD+ ID versus II (recessive model)			1.81	0.94–3.47	0.071	1.67	0.85–3.28	0.130

SNP, single-nucleotide polymorphism; VEGF, vascular endothelial growth factor.
[a]Statistical significance of risk association when $P \leq 0.05$.
[b]Statistical significance of no-risk association when $P \leq 0.05$. Adjusted for patient age, patient gender, diabetic status, donor age, donor gender, donor glomerular filtration rate, number of human leukocyte antigen mismatches, acute rejection episodes.

(HR = 2.42, $P = 0.0001$) and CA (HR = 1.83, $P = 0.009$) genotypes of −2578 C/A SNP with graft loss, whereas carrier −2578*CC genotype was shown to have a strongly protective association. We have observed prolonged graft survival for patients with the CC genotype of VEGF −2578 C/A SNP. mRNA quantification revealed better survival for patients with higher VEGF production.

Functional importance of several VEGF SNPs has been associated with production of VEGF protein by lipopolysaccharide-stimulated peripheral blood mononuclear cells.[12] We have studied the genetic association of 4 VEGF-A SNPs with long-term graft function and correlated the findings obtained with mRNA expression pattern for each genotype. Graft survival for CC and CA genotypes of VEGF −2578 C/A SNP was

Table 3. Comparison of transplant related characteristics between VEGF SNPs at −2578 C/A and −2549 Ins/Del genotypes

	VEGF -2578 C/A				VEGF -2549 Ins/Del			
Clinical characteristics	CC n = 61	CA n = 150	AA n = 61	P	DD n = 79	DI n = 135	II n = 58	P
Follow-up duration (mo)	51.9 ± 31.4	45.2 ± 25.9	46.4 ± 28.3	0.279	51.3 ± 28.9	45.0 ± 28.4	51.0 ± 28.7	0.202
Recipient age (yr)	38.8 ± 10.7	36.6 ± 11.4	41.6 ± 12.1	0.015[a]	38.8 ± 12.3	38.2 ± 11.2	37.4 ± 11.5	0.764
Recipient gender (male/female)	50/11	124/26	53/8	0.71	68/11	111/24	48/10	0.755
Recipient BMI (kg/m^2)	23.1 ± 1.2	23.0 ± 1.8	23.4 ± 1.6	0.36	23.0 ± 1.5	23.3 ± 1.8	22.9 ± 1.6	0.267
Diabetes (yes/no)	6/55	16/134	11/50	0.273	10/69	17/118	6/52	0.895
Donor age (years)	44.6 ± 10.3	44.5 ± 11.5	44.1 ± 10.1	0.964	44.1 ± 10.3	45.2 ± 11.1	43.0 ± 11.1	0.44
Donor gender (male/female)	18/43	40/110	13/48	0.572	23/56	36/99	12/48	0.529
Donor GFR (ml/min/1.73 m^2)	39.0 ± 6.4	39.4 ± 7.2	38.9 ± 6.1	0.831	39.8 ± 7.6	38.8 ± 6.1	39.2 ± 7.1	0.602
HLA mismatch	2.9 ± 1.0	3.0 ± 1.1	3.1 ± 1.0	0.539	3.1 ± 1.2	3.0 ± 1.0	2.7 ± 1.1	0.116
Delayed graft function (yes/no)	6/55	2/148	6/55	0.007[a]	3/76	9/126	2/56	0.528
Acute rejection (yes/no)	11/50	35/115	8/53	0.222	11/68	34/101	9/49	0.089

BMI, body mass index; GFR, glomerular filtration rate; HLA, human leukocyte antigen; SNP, single-nucleotide polymorphism; VEGF, vascular endothelial growth factor.
[a]Statistical significance when $P \leq 0.05$.

significantly better in comparison to the mutant AA genotype. Lemos *et al*. found a similar association with recipient but not donor VEGF SNPs (−2578 C/A and carrier haplotypes).[13] They suggested that renal allograft recipients with a genetic basis for high production of VEGF had significantly better graft survival in comparison to recipients with low VEGF production.[13] However, they did not evaluate mRNA levels for the significant −2578 genotypes, which we have shown in the present study. We observed a low level of VEGF mRNA expression for the mutant −2578 AA genotype in comparison to the wild-type −2578 CC genotype when the subjects with failed allograft were compared with subjects with a functioning allograft. The patterns of mRNA expression were noted to be high for wild-type genotypes and low for mutant genotypes among CAD subjects. This may suggest the effect of recipient-derived circulating VEGF levels on renal allograft survival.

In newborn mice, blocking of VEGF with antibodies resulted in abnormal glomerulogenesis.[14] Its role has been well documented in postinjury regeneration of damaged capillaries. VEGF has trophic and antiapoptotic effects on endothelial cells.[15] Experimental models have shown that decreased VEGF expression is associated with accelerated loss of peritubular capillaries and development of glomerulosclerosis and IF/TA.[16] In addition, VEGF has a pro-survival effect on renal tubular epithelial cells against calcineurin cytotoxicity.[7]

Some reports have suggested that local expression of VEGF within the graft during acute rejection can increase the recruitment and trafficking of allogeneic cells.[17] These findings may be explained by differential actions mediated by different VEGF receptors (VEGFR). Most pro-survival signals are mediated by VEGFR-2, whereas VEGFR-1 mediates pro-inflammatory and fibrogenic effects and downregulates VEGFR-2.[18,19] Recently, administration of exogenous VEGF in the systemic circulation was observed to result in decreased glomerular fibrosis and increased microvascular density in a hypertensive mouse model.[20] Granulocyte macrophage colony-stimulating factor−kindled monocyte and platelet-derived VEGF are predominantly involved in endothelial repair and regeneration, indicating that host-derived VEGF might be important.[21]

This study does have limitations. VEGF serum levels were not measured serially to definitely demonstrate an association between increased production of VEGF and graft outcome. Another limitation is the absence of a protocol biopsy and donor-specific antibody levels (due to the high cost in India) to confirm or establish the cause of CAD and to rule out an etiological impact in the role of VEGF. The strengths of our study are its prospective nature, a uniform protocol for immunosuppression, and a histopathological examination of tissues.

We conclude that genetic variability in VEGF production may influence allograft survival. This suggests an important role for VEGF in the pathophysiology of chronic allograft injury, and could be a target for potential intervention.

DISCLOSURE

All the authors declared no competing interests.

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AUTHORSHIP

SP, MRP, SA, and NP participated in research design. SP, MRP, SA, RMJ, and NP participated in the writing of the paper. SP, MRP, SA, and NP participated in the performance of the research. SP, MRP, SA, RMJ, and NP contributed new reagents or analytic tools. SP, MRP, SA, RMJ, and NP participated in data analysis.

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