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Vascular endothelial growth factor gene polymorphisms in North Indian patients with end stage renal disease

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ABSTRACT

Context: Vascular endothelial growth factor (VEGF) is involved in the development and differentiation of the vascular system. VEGF is expressed constitutively by epithelial cells from embryonic to adult kidneys and may play a key role in progression of kidney diseases. It is required for the growth and proliferation of glomerular and peritubular endothelial cells. In the kidney VEGF expression is prominently found in glomerular podocytes and in tubular epithelial cells, while VEGF receptors are mainly seen on preglomerular, glomerular, and peritubular endothelial cells.
Objectives: We have investigated the role of *VEGF* gene polymorphisms (−2578C/A,−2549 18 bp I/D, −1154 G/A and +936 C/T) as a susceptibility marker for end stage renal disease (ESRD).
Participants and methods: We genotyped VEGF gene polymorphism in three hundred patients and three hundred and fifty ethnically matched unrelated healthy controls free from any renal disease. These markers were studied using ARMS-PCR and PCR-RFLP methods. Patients were categorized on the basis of the histo-pathological subtypes into chronic glomerulonephritis (CGN = 109), hypertensive nephrosclerosis (HTN = 106) and chronic interstitial nephritis (CIN = 60).
Results: **VEGF −2578C and −2549D alleles were found to be ESRD causative alleles**. It was observed that there was significant differences in the frequencies of the **T** allele of +936C/T polymorphism among CGN, HTN and CIN respectively. VEGF −1154**AA** genotype and **A** allele were associated significantly with CGN. T–G–A–D, T–A–C–I,C–G–A–D,C–A–C–D,C–G–C–I,C–A–A–D and T–G–C–D were seven haplotypes concurred in all the ESRD patients irrespective of underlying disease. While C–G–C–D & C–G–A–I haplotypes showed risk association in CGN & CIN, C–A–C–I was observed to play predisposing role in HTN.
Conclusion: The results highlight the role of studied VEGF polymorphisms in end stage renal disease at large and subsequently in the three primary kidney diseases among the North Indian population.

1. Introduction

Kidney is a highly vascularized organ. Vascular endothelial growth factor (VEGF) which is constitutively expressed by epithelial cells from embryonic to adult kidneys might play a key role in progression of kidney diseases. VEGF expression has been demonstrated in the podocytes [1] and mesangial cells in kidneys of patients with glomerulonephritis [2]; VEGF receptors have been detected in the endothelium of glomeruli and peritubular capillaries [2] which prevent progression of kidney diseases. It acts as a survival factor that allows cells to survive and proliferate under conditions of extreme stress, both in vivo and in vitro [3]. Administered in case of acute glomerular injury VEGF causes induction of glomerular repair & resolution of glomerulonephritis associated with stimulation of angiogenesis & vascular remodeling [4]. It is a potent regulator of vasculogenesis and angiogenesis which may amplify acute inflammatory reactions. Transcription factor is activated on exposure of endothelial cells and macrophages to VEGF. Additional abnormalities besides hyper proliferation of blood vessels are observed upon exposure of tissues to high VEGF concentration [5]. Upon reduction or total inhibition of VEGF supply impaired angiogenesis may occur which leads to the inhibition of organ development [6].

The VEGF gene is located on chromosome 6p 21.3 [7] which is highly polymorphic, especially in the promoter region, 5' untranslated region (UTR) and 3'UTR [8,9].The polymorphisms in these regions have been reported to be associated with VEGF levels [9,10]. The −2578 C/A, −1154 G/A, −2549 I/D polymorphisms are reported in the 5'UTR of promoter region while the +936 C/T

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polymorphism in the 3'UTR of promoter region. VEGF is expressed constitutively in the adult kidney and over expressed in hypoxic and inflammatory renal disease. Genetically controlled variation in VEGF production may influence susceptibility to progression of kidney diseases. Present study was aimed to investigate the roles of −2578C/A, −1154 G/A, −2549 18 bp I/D and +936 C/T polymorphisms and their haplotypes among ESRD patients, subsequently studying their incidence in primary kidney diseases namely chronic glomerulonephritis (CGN), chronic interstitial nephropathy (CIN) and hypertensive nephrosclerosis (HTN). This is the first study evaluating these polymorphisms among the North Indian population.

2. Materials and methods

2.1. Subjects

Three hundred ESRD patients [Male = 244 (81%), Female = 56 (19%)] and three hundred and fifty healthy age and sex matched North Indians were included as controls [Male = 280 (80%), Female = 70 (20%)] in this study. The inclusion criteria for patient selection was constantly elevated serum creatinine level above normal range (ranging from 3.4 to 15.8 mg/dl) or creatinine clearance <15 ml/min/1.73 m^2 and were recommended for renal transplantation. We have used Cockcroft Gault for calculation of creatinine clearance. All the patients selected for the study were on regular hemodialysis. Patients were considered hypertensive if they were on anti hypertensive medication or if systolic or diastolic blood pressure was $\geqslant$140 or 90 mm Hg (blood pressure was measured twice with a mercury sphygmomanometer, in supine position, after at least 10 min of rest, and results were averaged). For each patient, various information like age, gender, urinary protein level, blood urea nitrogen, blood pressure and complete lipid profile were collected. The type of chronic kidney disease was established by doing ultrasound and/or CT scan of the kidneys followed by histopathological evaluation of the renal biopsy specimen. Patients were categorized on the basis of the histopathological subtypes intochronic glomerulonephritis (CGN = 109), chronic interstitial nephritis (CIN = 106), hypertensive nephrosclerosis (HTN = 60). All patients with diabetic nephropathy were excluded from the study.

Approximately 85% of the patients were male hence care was taken to include more number of male controls in order to rule out gender bias. Controls with risk factors like family history of hypertension, diabetes mellitus and hyperlipidemia were excluded from the study. The criterion of defining control sample as normal was totally based on the absence of any kidney disease determined from the serum creatinine level. The mean creatinine level of controls was found to be 0.74 ± 0.23 while that of patients was 7.17 ± 3.1.Similar kind of proforma as for patients was also filled for controls. The study was performed as per the ethical standards laid down by the Declaration of Helsinki. Informed written consent was obtained from both the patients and controls prior to their inclusion in the study. Further the study was approved by the institute ethics committee.

2.2. Sample collection, DNA extraction and VEGF Genotyping

Blood samples for measuring the serum biochemical parameters were obtained in the morning after eight hours of fasting. For DNA extraction, 3.0 ml of venous blood from each study subject was collected in an EDTA vial. Genomic DNA was obtained using genomic DNA extraction kit from Quiagen (Brand GMbH and Co KG, Cat # 51104).

Amplification of the four regions of the VEGF gene containing the polymorphisms −2578C/A, −1154G/A, −2549 I/D, and +936C/T were carried out in a thermal cycler (Mastercycler gradient; Eppendorf, Hamburg, Germany).Genotype analyses of −2578 C/A and −1154G/A were based on ARMS PCR and RFLP was used for +936C/T polymorphisms as explained by Papazoglou et al. [11]. Primers used for the genotyping purpose is shown in supplementary table (Supplementary Table 1). In case of the VEGF + 936C/T, the PCR product was digested overnight with NlaIII restriction enzyme (New England BioLabs, Beverly, MA,USA). The VEGF 936T allele was cut into two fragments of 122 bp and 86 bp, whereas the VEGF 936C allele remained uncut with a length of 208 bp (Fig. 1).

The −2549 I/D polymorphism was genotyped using a common set of primers as mentioned by Buraczynska et al. [12]. PCR and RFLP products were run on 2% agarose and visualized using ethidium bromide (Fig. 2). **The genotyping was done in a double blind manner by two investigators and after decoding nearly 15% samples required re-genotyping.**

2.3. Statistical analysis

The sample size for both the patients and controls was calculated under the guidance of a statistician. Differences in the VEGF genotypes, allele frequencies and haplotypes between the study and control groups were analyzed with the Fisher exact test and p values <.05 were considered statistically significant. Bonferroni correction to the p-value was applied. The Odds Ratio (OR) was used as a measure of the strength of association between genotypes, allele frequencies and haplotypes. Haplotypes were generated using Arlequin software and statistical analysis was done using SPSS software version 13.Allele frequencies were calculated as the number of occurrences of the test allele in the population divided by the total number of alleles.

3. Results

Demographic characteristics and baseline laboratory data are provided in Table 1. Two most important renal function

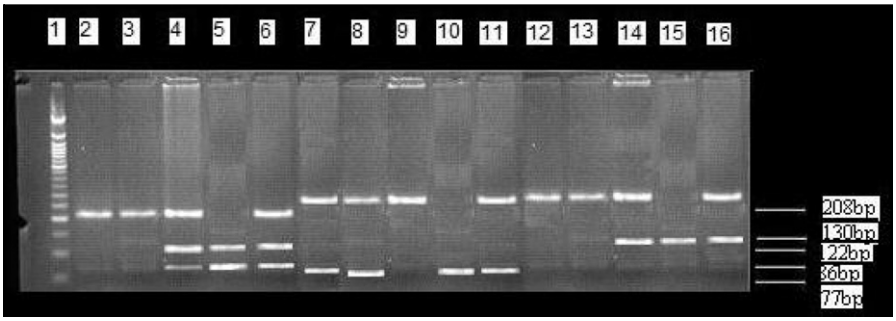

Fig. 1. Vegf +936C/T polymorphism Lane 1–50 bp ladder, Lane 2,3-TT genotype, Lane 4 & 6-CT genotype, Lane 5-CC genotype; VEGF −2578C/A polymorphism Lane 7,8-CA genotype, Lane 9,10-AA genotype, Lane 11,12-CC genotype; VEGF -1154G/A polymorphism Lane 13,14-AA genotype, Lane 15,16-GA genotype.