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ORIGINAL ARTICLE

# VEGF Promoter Region 18-bp Insertion-Deletion Polymorphism in Sickle Cell Disease Patients with Microalbuminuria: A Pilot Study

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**Abstract**

*Purpose* Vascular endothelial growth factor (VEGF) is a potent inducer of micro vascular permeability thus leading to nephropathy. Insertion/deletion (I/D) polymorphism of 18 bp at − 2549 position in VEGF gene causes increased transcription leading to increased production of VEGF. Thus, we aimed to associate I/D polymorphism of the 18 bp fragment at − 2549 position of the promoter region of VEGF gene with sickle cell nephropathy (SCN).

*Methods* This observational analytical case control study included 30 subjects each of SCN, sickle cell disease (SCD) without nephropathy and the control group. The subjects were assessed for various hematological and biochemical parameters. Further, 18 bp I/D polymorphism of VEGF gene in all three study groups was assessed by polymerase chain reaction followed by electrophoresis and compared.

*Result* Though increased frequency of both DD genotype and D allele was found in SCN compared to SCD and control, only frequency of D allele was found to be significantly higher ($p$ = 0.04). D allele posed marginal risk of microalbuminuria in SCD subjects compared to controls (OR = 2.11) as well as to SCD without MA subjects (OR = 1.84).

*Conclusion* D allele in I/D polymorphism in the promoter region of VEGF gene may be associated with marginal increase in risk of susceptibility to sickle cell nephropathy.

**Keywords** Sickle cell disease · Sickle cell nephropathy · VEGF · Polymorphism

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## Introduction

Sickle cell disease (SCD) is characterized by the production of abnormal sickle shaped red cells, variable degree of hemolytic anemia as well as acute and chronic tissue damage triggered by vaso-occlusion [1]. The molecular basis suggests the transversion of A-to-T in the codon for sixth position in the β-hemoglobin gene (HBB). This mutation results in the replacement of the normal polar glutamic acid residue by a non-polar valine residue (glu6 val) and substitution of normal HbA β-globin chains by HbS β-globin chains [2].

Several structural and functional abnormalities of the kidney are observed in patients presenting sickle cell anemia and the related hemoglobinopathies. Sickle cell nephropathy is a severe complication with high prevalence and is also an independent factor of mortality in SCD patients [3]. It manifests in different forms including

glomerulopathy, proteinuria, hematuria, and tubular defects and frequently results in end-stage renal disease (ESRD) [4]. Hemoglobinuria is found to associate independently with glomerular injury and CKD and has been reported in approximately 15–42% of SCD patients [5, 6]. The renal consequences of SCD manifest in early childhood and include different portions of the nephron. In SCN patients, renal function and the presence of albuminuria are believed to significantly correlate with the hemolytic markers, namely reticulocyte count and bilirubin [7, 8]. Glomerular filteration rate (GFR) increases during the early nephropathy. However, it tends to decrease in the second decade of life with the progression and development of SCN [9].

Vascular permeability factor or VEGF, produced by podocytes is one of the most potent inducers of micro vascular permeability and it positively correlates with urinary albumin creatinine ratio. Insertion/deletion (I/D) polymorphism of the 18 bp at − 2549 position of the promoter region in VEGF gene has been implicated in a number of diseases with angiogenic basis and hence is of particular interest. Increased risk of microvascular complications as nephropathy has been previously associated with I/D polymorphism in VEGF gene [10–12]. The polymorphism at − 2549 position alters the expression of gene, thereby affecting the quantity of protein leading to increased serum level of VEGF and increased risk of nephropathy [13–15].

There is paucity of data assessing VEGF I/D polymorphism in sickle cell nephropathy subjects. To the best of our knowledge no authors have assessed this particular polymorphism in Indian subjects with sickle cell nephropathy. Thus, we aimed to assess insertion/deletion polymorphism of the 18 bp fragment at − 2549 position of the promoter region of VEGF gene in subjects with early sickle cell nephropathy.

## Materials and Methods

### Design and Subject

This hospital based observational, Analytical case control pilot study was conducted in Sickle Cell Institute, Department Of Biochemistry, Pt. J. N. M. Medical College, Raipur (C.G.). Sickle cell disease patients with early nephropathy as defined by microalbuminuria (ACR: 30–300 mg/g of creatinine), SCD patients without nephropathy (ACR: < 30 mg/g of creatinine) and apparently healthy normal subjects (control) were respectively designated as group I, II, and III, each consisting of 30 subjects. The study included subjects aged between 1 to 18 years of either gender. Subjects aged > 18 years and history suggestive of other causes of nephropathy (DM, HT, HCV, HIV etc.), pregnancy and any associated malignancy were excluded from the study.

## Methods

After obtaining informed written consent from parents and an assent from subjects, medical history was elicited. 5 ml of fasting venous blood was obtained after overnight fasting. Two ml of blood was separated in a vial containing EDTA for performing haematological test and 2 ml of blood without any anticoagulant was used for performing biochemical test. The diagnosis of the of sickle cell disease (HbSS), sickle cell trait (HbAS) and normal controls (HbAA) was established by performing hae moglobin electrophoresis and High performance liquid chromatography (HPLC) using Hb Variant ® (Bio-Rad Laboratories, Hercules, CA, USA). for estimation of various biochemical analyses. Serum urea, creatinine, total protein, albumin and other relevant biochemical parameters in the blood samples were detected by clinical chemistry analyzer, 'ILab 650®, (Werfen®, Belgium). Ten ml of early morning spot urine sample obtained for urinary microalbumin was estimated by nephelometer(Beckman Coulter®, Germany, CA, USA) using Beckman coulter microalbumin kit (ref. OSR6167) and creatinine was estimated on clinical chemistry analyser in 10 X dilution.

### Determination of VEGF Genotype

In present study, I/D polumorphism of 18 bp fragment at − 2549 position of the promoter region in VEGF gene was selected. It was located at the promoter region of VEGF gene based on its functional nature. The I/D polymorphism was analyzed using following primers: forward 5′-GCTGAGAGTGGGGCTGACTAGGTA-3′ and reverse 5′-GTTTCTGACCTGGCTATTTCCAGG-3′. The amplification was done by PCR and the products were separated by electrophoresis on 2.5% agarose gel stained with ethidium bromide. For the VEGF I/D polymorphism two bands were observed, 211 bp and 229 bp for D and I alleles respectively (Fig. 1).

### Statistical Analysis

The data were analyzed using the statistical package SPSS® for windows™ (version 16.0 IBM™ Corp. NY) and Microsoft Excel™ 2007, Microsoft® Inc USA. Kolmogorov–Smirnov analysis was performed to assess the linearity of data. Data was expressed as mean ± SD or Median (range) depending upon distribution. Statistical differences between categorical data like gender, genotype

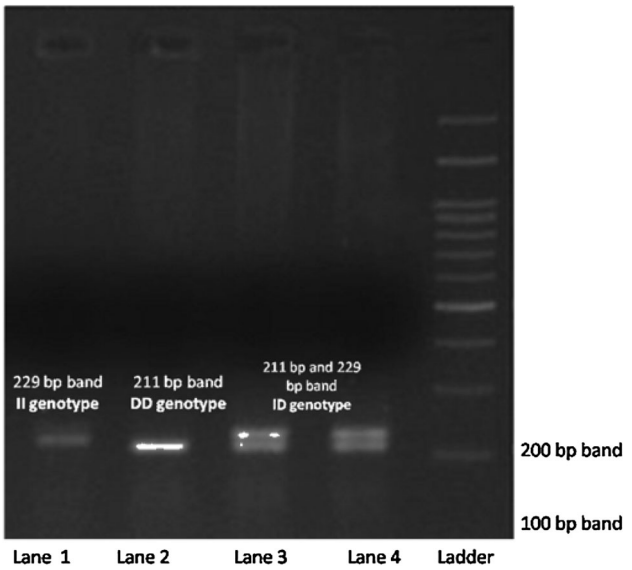


**Fig. 1** Agarose gel electrophoresis run of PCR products for VEGF Gene. Lane 1:229 bp band for II genotype, Lane 2 was showing 211 bp band DD genotype and lane 3 and 4 showing both 211 bp and 229 bp bands for ID genotype

distribution was tested using Chi square test and fischer's exact test. Quantitative variables were tested using ANOVA. Post hoc analysis was performed by tukey's HSD test in case of paramtetric data and Kruskal–Wallis followed by Dunn test in case of non-parametric data (Urinary microalbumin and ACR). *P* values $\leq 0.05$ was considered as statistically significant.

### Observations

Table 1 presents the baseline characteristics of the study subjects and controls. The three groups were matched for in terms of age ($p = 0.4$) and gender ($p = 0.8$). Significant difference in hemoglobin ($p < 0.0001$), RBC ($p < 0.0001$), MCHC ($p = 0.048$), hematocrit ($p < 0.0001$), MCV ($p = 0.06$), RDW ($p < 0.0001$), WBC ($p < 0.0001$), ($p = 0.03$), AST ($p = 0.009$), ALT ($p = 0.004$), ALP ($p < 0.0001$) and Urinary ACR ($p < 0.001$) was noted amongst groups.

Further on post hoc analysis, significant decrease in hemoglobin, RBC, MCHC and haematocrit was observed in SCD with MA and SCD without MA and control groups. However, RDW, WBC and AST levels were found to be significantly higher in SCD with MA and SCD subjects without MA in comparison to control group. Platelet count and ALT levels were found to be significantly decreased in SCD with MA against controls. Urimary microalbumin and urinary ACR was found to be significantly higher in SCD with MA compared to both SCD without MA and Control, while no such difference was detected between SCD with MA and SCD without MA.

Genotype distribution between study groups was assessed using Chi square test. No significant difference was found between study groups regarding frequency of various genotypes. Further No significant difference was detected on comparison of genotype frequency between controls and SCD with MA and SCD without MA. Though frequency of DD genotype was higher in SCD with MA group compared to Control ($p = 0.20$) and SCD without MA group ($p = 0.82$), the difference failed to reach statistical significance. Further Frequency of D allele was found to be significantly higher in SCD with MA group compared to control group ($p = 0.04$). No such significance was detected between SCD with MA and SCD without MA despite the frequency of D allele being higher in the later.

D allele was found to pose margianlly increased risk of microalbuminuria in SCD subjects compared to controls (OR 2.11 CI 0.01–4.38) as well as to SCD without MA subjects (OR 1.84, CI 0.89–3.82) (Table 2).

## Discussion

In present pilot study DD polymorphism of 18 bp fragment at − 2549 position of VEGF gene was analyzed. We found that DD genotype in SCD with MA subjects was significantly higher than SCD subjects without MA as well as controls. Control group here represents the baseline frequency of genotypic distribution among normal population. Further findings suggested that SCD subjects with MA consisting of DD genotype were at moderately increased risk of sickle cell nephropathy. The findings of our pilot study are based on relatively small sample size and hence the statistical significance is also borderline.

Vascular endothelial growth factor (VEGF) causes angiogenesis and increases vascular permeability, but in SCN endothelial dysfunction is observed. This fact is supported by the increased levels of microalbumin, and ACR significantly. Endothelial dysfunction in the glomerular endothelium promotes an activated, proinflammatory and procoagulant endothelial phenotype; inflammatory mediators such as endothelin-1 and tumour necrosis factor from the glomerular endothelium in turn undertakes receptors on podocytes, thereby causing podocyte injury and proteinuria [16]. VEGF has been demonstrated in human kidney in podocytes, proximal and distal tubular cells and VEGF receptors are found in pre, post and glomerular region and on masengial cells [17].

Human VEGF gene is highly polymorphic in nature and I/D Polymorphism of 18 bp fragment at − 2549 of the promoter region is of great significance owing to its involvement in development of many diseases, especially

**Table 1** Baseline characteristics of study subjects

| Characteristics | SCD with MA (n = 30) | SCD without MA (n = 30) | Control (n = 30) | *P* Value |
|---|---|---|---|---|
| Age (years) | 13.1 ± 4.03 | 11.83 ± 3.49 | 15.1 ± 2.33 | 0.4 |
| Gender | | | | |
| Male | 18 | 17 | 20 | 0.8 |
| Female | 12 | 13 | 10 | |
| Hb (gm/dl) | 9.0 ± 1.9 | 10.2 ± 2.8 | 13.7 ± 1.8[a,b] | < 0.0001 |
| RBC (million cells/µl) | 2.84 ± 0.62 | 3.13 ± 1.14 | 4.75 ± 0.67[a,b] | < .0001 |
| MCV (fl) | 88.0 ± 10.8 | 86.9 ± 13.3 | 82.1 ± 7.4 | 0.06 |
| MCHC (g/dl) | 34.7 ± 3.4 | 34.0 ± 3.2 | 35.7 ± 2.5[a,b] | 0.048 |
| MCH (pg/cell) | 30.7 ± 4.6 | 30.0 ± 4.5 | 29.3 ± 3.4 | 0.4 |
| RDW (in %) | 17.3 ± 3.6 | 17.6 ± 3.5 | 13.9 ± 1.1[a,b] | < 0.0001 |
| WBC (cells/mm$^3$) | 11.34 ± 3.66 | 10.4 ± 3.5 | 7.07 ± 1.39[a,b] | < 0.0001 |
| Haematocrit (%) | 25.66 ± 5.55 | 30.1 ± 7.6 | 38.63 ± 5.44[a,b] | < 0.0001 |
| Platelet count (100/µL) | 311.9 ± 149.5 | 261.4 ± 100.8 | 233.3 ± 53.6[a] | 0.03 |
| AST (U/L) | 47.66 ± 35.39 | 51.1 ± 48.4 | 23.2 ± 23.2[a,b] | 0.009 |
| ALT (U/L) | 44.16 ± 21.82 | 33.1 ± 20.6 | 26.53 ± 18.98[a] | 0.004 |
| ALP (IU/L) | 104.86 ± 30.58 | 96.4 ± 34.1 | 65.46 ± 15.56 | < 0.0001 |
| Serum Na (mmol/L) | 141.3 ± 3. | 140.1 ± 2.2 | 140.9 ± 4.89 | 0.26 |
| Serum K (mmol/L) | 4.16 ± 4.16 | 4.1 ± 0.1 | 4.07 ± 0.44 | 0.4 |
| Urinary microalbumin (mg/dl) | 3.16 (2.11–27.1) | 0.30 (0.112–1.51)[a] | 0.29 (1.1–0.12)[a] | < 0.0001 |
| Urinary creatinine (mg/dl) | 66 (25–144.5) | 48.7 (10–362.5) | 40.75 (15–123) | 0.44 |
| Urinary ACR (mg/g of Cr) | 80 (19.06–299.04) | 7.5 (0.4–27.8)[a] | 5.8 (1.4–25)[a] | < 0.001 |

[a]$p < 0.05$ versus SCD with MA

[b]$p < 0.05$ versus SCD without MA

**Table 2** Genotype distribution in study groups

| | Genotypes | | | Alleles | | Odd's ratio (95% CI) | |
|---|---|---|---|---|---|---|---|
| | DD | ID | II | D | I | | |
| SCD with MA | 14 (46.66)[*] | 10 (33.33) | 6 (20) | 38[a] | 22 | 2.11 (1.01–4.38) | 1.84 (0.89–3.82) |
| SCD without MA | 9 (30)[**] | 11 (36.66) | 10 (33.33) | 29[b] | 31 | 1.14 (0.55–2.34) | Baseline |
| Control | 9 (30) | 9 (30) | 12 (40.0) | 27 | 33 | Baseline | 0.87 (0.42–1.79) |

*$p = 0.20$, **$p = 0.82$, [a]$p = 0.04$, [b]$p = 0.71$

those based on angiogenic origin [18]. Potential association of VEGF gene polymorphisms with nephropathy and retinopathy has been investigated in previous studies. Findings done by the studies Amle et al. [10] and Yang et al. [11] goes in favor of our study that DD genotype may pose borderline risk for nephropathy. Possible explanation given is that D allele is said to be associated with increased transcription of VEGF compared to I allele leading to raised serum levels. Effects of DD genotype has also been found to be associated with increased serum levels of VEGF by Buraczynska et al. [12]. A positive association between I/D polymorphism of VEGF gene in patients with type 2 diabetes and diabetic peripheral polyneuropathy, another microvascular complication, has been suggested by Stoian et al. [19]. VEGF gene was also found to be implicated in the development of diabetic retinopathy [20]. Bleda et al. [21] also provided that VEGF polymorphisms are involved in late vascular complications in type 2 diabetic patients.

Though VEGF polymorphism may be regarded as borderline risk factor as found in our study, various other associated conditions are also known to contribute to progress of sickle cell nephropathy. Co-inheritance of β thalassemia is one such factor and have been regarded as one of the known predictor of advanced renal insufficiency [22]. α Thalassemia though has been regarded as providing

protection in urinary concentration defect by favorably altering the rheological factors in dose dependent manner but exact role of these co-inheritance is yet to be studies in glomerual defects [23]. Hemoglobin F also have been proved protection by altering the sickling in SCA subjects and thus reduces the incidence of microalbuminuria [24]. Some variants of heme oxygease 1 (*HMOX1*) as well as *APOL1* are amongst the factors implicated in pathophysicology of SCN though their roles have not yet been clearly established due to conflicting results in different studies [25, 26].

The possible mechanism to support current borderline risk posed by 18 bp insertion deletion polymorphism at − 2549 position of promoter region of VEGF gene can be by promoter shortening which further causes increased transcription of VEGF protein leading to increased serum VEGF levels [10, 12]. Although, the effect of VEGF polymorphism in SCD with MA subjects was not significant, a limitation of our study is inadequate number of sample size and thus possible type 2 error. Thus in conclusion borderline significance observed in difference in allele frequency, though indicating moderately increased risk for sickle cell nephropathy in sickle cell subjects, this may or may not indicate a true relationship, and this study only provides a basis for studying a larger number of cases.

**Compliance with Ethical Standards**

**Conflict of interest** All authors declare that there is no conflict of interest related to the work.

**Ethical Approval** The study was conducted in blood and urine samples received from human subjects. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and were approved by institutional ethical committee of Pt. J. N. M. Medical College and Hospital, Raipur, CG and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

**Informed Consent** Written informed consent was obtained from the parent or the legal guardian and assent was obtained from the subjects for all individual participants included in the study.

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