

## **VEGF gene promoter polymorphisms are associated with diabetic foot ulcer.**

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### **Abstract**

We investigated the relationship between the -460T/C and -1154G/A polymorphisms in the promoter region of the vascular endothelial growth factor (VEGF) gene and diabetic foot ulcer (DFU) in a northern Han Chinese population. A total of 209 type 2 diabetes mellitus (T2DM) patients were examined in this hospital-based case-control study. Polymerase chain reaction-restriction fragment length polymorphism was used to detect polymorphisms at the -460T/C and -1154G/A loci of the *VEGF* gene promoter in 94 healthy controls and 209 T2DM patients, including 112 cases of T2DM alone and 97 cases of T2DM with DFU. Genotype and allele frequencies in each group were compared. Patients and controls exhibited no significant differences in polymorphisms at the -1154G/A locus of the *VEGF* gene promoter. However, at the -460T/C locus, the C allele was observed with significantly lower frequency in DFU patients than in patients with T2DM alone (T/C + C/C vs T/T,  $P=0.0109$ ,  $OR=0.4708$ ,  $CI: 0.2625-0.8444$ ). The C allele was also observed significantly less frequently in DFU patients than in control group patients ( $P=0.0037$ ,  $OR=0.4784$ ,  $CI: 0.2891-0.7916$ ). This study demonstrated that the C allele of the -460T/C polymorphic locus is present at a lower frequency in DFU patients than in other individuals. This may reflect the protective role of this allele in increasing angiogenesis in patients.

**Keywords:** Diabetic foot ulcers, Polymorphic loci, Vascular endothelial growth factor.

*Accepted on September 14, 2016*

### **Introduction**

The term of diabetic foot ulcer (DFU) is referred to the foot pain, deep skin ulcers, gangrene, and other lesions occurring as a result of diabetes and its complications [1]. DFU is associated with not only distal nerve abnormalities of the lower extremities but also foot infections, ulcers, and deep tissue damage due to various degrees of peripheral vascular disease [2,3]. DFU is one of the main causes of disability and death among diabetic patients. In developed countries, the incidence of DFU among diabetic patients is approximately 4-10% [4,5]. DFU occurs mainly because of the long-term poor hyperglycemic control, leading to not only the stenosis and occlusion of capillaries and large, medium, and small blood vessels, but also to blood flow disorders [6]. As a result, ischemic injury to foot neurons occurs, and sensory, motor, and autonomic nerves are damaged. Currently, common therapeutic approaches for treating DFU include targeting the causes and mechanisms of the disease, transplanting adult, embryonic, and hematopoietic stem cells into patients, and promoting angiogenesis [3].

Studies have demonstrated that a variety of growth factors are effective for treating late-stage complications of diabetes. Among these factors, vascular endothelial growth factor (VEGF) has attracted a great deal of attention [7]. VEGF, known as vascular permeability factor, is the most powerful

known endothelial cell-specific mitogenic and angiogenic factor. VEGF specifically stimulates endothelial cell proliferation, promotes endothelial cell migration, and participates in the formation of new blood vessels [8]. VEGF promotes mitotic activity mainly through its interactions with 2 receptors, VEGF receptor-1 (VEGFR-1) and VEGF receptor-2 (VEGFR-2). In particular, VEGFR-1 plays a role in vascular permeability, while VEGFR-2 plays a role in angiogenesis [9]. Neovascularization can provide the necessary oxygen and nutrients for granulation tissue formation and wound healing [10]. Given the existence of microcirculatory disorders in DFU, studies have suggested that this disease can be prevented and treated by regulating VEGF expression [11].

The *VEGF* gene is highly polymorphic. At present, more than 30 loci with single-nucleotide polymorphisms (SNPs) have been identified in *VEGF* gene [12,13]. These SNPs may affect VEGF expression and/or activity, leading to differences in the regulation of angiogenic functions by VEGF and causing individuals to exhibit different susceptibilities to DFU [14]. These sites may be novel genetic markers for analyzing the association between VEGF and DFU. Of the polymorphic loci of VEGF, the -460T/C and -1154G/A sites in the promoter region may influence VEGF protein expression and thereby affect the biological functions of VEGF [15]. In this study, we examined how the -460T/C and -1154G/A SNPs in the

promoter region of the *VEGF* gene relate to genetic susceptibility to DFU in a northern Han Chinese population.

## Material and Methods

### Research subjects and grouping

A total of 209 type 2 diabetes mellitus (T2DM) patients with (97) or without (112) DFU treated at the First Affiliated Hospital of Harbin Medical University between March 2009 and March 2011 were selected. In addition, 188 healthy individuals who received physical examinations at the hospital were included as a control group. The subjects were all from the northern Han Chinese population. There were no blood relationships between any of the examined individuals. All participants were provided informed consent. After personal information was recorded, 5 mL of venous blood was drawn into a tube containing ethylenediaminetetraacetic acid and the blood samples were stored at -20°C until genomic DNA extraction.

### Diagnostic criteria

Patients were diagnosed according to the Diabetes Classification and Diagnostic Criteria issued by the American Diabetes Association. The severity of foot ulcers was determined using the Wagner system.

### Detection of polymorphisms in the *VEGF* promoter

The polymerase chain reaction-restriction fragment length polymorphism technique was performed using genomic DNA

from peripheral blood leukocytes as a template. Based on the nucleotide sequence recorded in GenBank for the *VEGF* gene (GenBank accession No.: AF095785.1), genotyping primers were designed to amplify the -460T/C and -1154G/A polymorphic loci in the promoter region of *VEGF*. Primers were synthesized by Shanghai Bioengineering Co., Ltd. (Shanghai, China). The upstream and downstream primers of the -460T/C region were 5'-TGT GCG TGT GGG GTT GAG CG-3' and 5'-CGT GCG GAC AGG GCC TGA-3', respectively, whereas the upstream and downstream primers of the -1154G/A region were 5'-TCC TGC TCC CTC GCC AAT G-3' and 5'-GGC GGG GAC AGG CGA GCA TC-3', respectively. Amplification products were subjected to 2% agarose gel electrophoresis. Genotypes were determined based on whether target sequences were amplified.

### Statistical analysis

The SPSS 11.0 statistical software package was used for statistical analysis of the study data (SPSS, Inc., Chicago, IL, USA), with  $P < 0.05$  regarded as a statistically significant difference. In this study, t-tests were used to assess measurement data. Strength of association between different groups and alleles or genotypes of *VEGF* gene polymorphism was estimated using odds ratios (OR) and 95% confidence intervals (CI). Levels of significance were determined using contingency tables by either Chi-square or Fisher exact analysis.

**Table 1.** Clinical indicators for DFU patients, patients with T2DM alone, and normal controls.

	Normal controls [N (%)]	DFU patients [N (%)]	Patients with T2DM alone [N (%)]
<b>Gender</b>			
Male	102 (54.3)	45 (46.4)	69 (62.3)
Female	86 (45.7)	52 (53.6)	43 (37.7)
Age (years)	53 ± 10.2	54 ± 10.3	56 ± 10.4
<b>Family history of diabetes</b>			
Yes	50 (26.6)	58 (59.8)	71 (63.4) <sup>a</sup>
No	138 (73.4)	41 (40.2)	82 (36.6)
Duration of diabetes (years)	-	6.9 ± 5.2	14.8 ± 8.9 <sup>b</sup>

<sup>a</sup>Patients with a family history of diabetes had significantly higher frequencies of DFU compared with T2DM alone;  $P = 0.03$

<sup>b</sup>Prevalence of DFU relative to T2DM alone increased significantly as the duration of diabetes increased;  $P < 0.001$ , 95% CI: 6.34-9.42.

## Results

Table 1 summarizes the clinical indicators of all participants. SNPs at the -460T/C and -1154G/A loci of the *VEGF* gene promoter are presented in Table 2. Comparisons of allele and genotype frequencies at these loci revealed that DFU patients significantly differed from controls at the -460T/C locus of the *VEGF* gene promoter. At this locus, the C allele was observed

at a significantly lower frequency among DFU patients than among patients with T2DM alone (T/C + C/C vs T/T,  $P = 0.0109$ , OR = 0.4708, CI: 0.2625-0.8444), suggesting that the C allele had protective effects against DFU (Table 2). At the -460T/C locus, carriage of the C allele also occurred at a significantly lower frequency among DFU patients than among normal controls ( $P = 0.0037$ , OR = 0.4784, CI: 0.2891-0.7916).

DFU patients, patients with T2DM alone, and normal controls did not significantly differ (Table 2) in allele and genotype frequencies at the -1154G/A polymorphic locus.

**Table 2.** Genotype and allele frequency distributions at the -460T/C and -1154G/A loci of the VEGF promoter for healthy controls (C), patients with T2DM alone (P), and DFU patients with T2DM.

	C [N (%)]	P [N (%)]	DFU [N (%)]	P-value		
				DFU/ C	DFU/ P	P/C
<b>-460T/C</b>						
Genotype						
T/T	98 (52.1)	63 (56.3)	71 (73.2)			
C/T	76 (40.5)	42 (37.4)	22 (22.7)	0.000 6	0.010 9	0.488 6
C/C	14 (7.4)	7 (6.3)	4 (4.1)			
Allele						
T	136 (72.3)	168 (75.0)	164 (84.5)	0.003 7	0.016 2	0.540 9
C	52 (27.7)	56 (25.0)	30 (15.5)			
<b>-1154G/A</b>						
Genotype						
G/G	96 (51.1)	66 (58.9)	57 (58.8)			
G/A	82 (43.1)	39 (34.8)	36 (37.1)	0.216 8	0.980 6	0.186 2
A/A	10 (5.8)	7 (6.3)	4 (4.1)			
Allele						
G	137 (72.9)	171 (76.3)	150 (77.3)	0.314 7	0.812 8	0.419 7
A	51 (27.1)	53 (23.7)	44 (22.7)			
DFU/C (C/T + C/C vs T/T): P = 0.0006, OR = 0.3987, 95% CI: 0.2341-0.6793						
DFU/C (C vs T): P = 0.0037, OR = 0.4784, 95% CI: 0.2891-0.7916						
DFU/P (C/T + C/C vs T/T): P = 0.0109, OR = 0.4708, CI: 0.2625-0.8444						
DFU/P (C vs T): P = 0.0162, OR = 0.5488, 95% CI: 0.3352-0.8984						

**Discussion**

DFU is a complex multifactorial disease that involves multiple related genes [16]. Previous studies have indicated that genetic factors play the important roles in the pathogenesis of DFU [5]. Additionally, various levels of VEGF expression have been observed in serum samples from patients from different regions who suffer from diabetic complications [17]. Thus, evidence-based medicine has suggested that VEGF plays a role in the occurrence and development of DFU. Fu et al. [18] demonstrated that sustained hyperglycemia can lead to vascular disease and that the basis of vascular disease is endothelial cell damage. Thus, vascular disease-induced damage causes hypoxia, thereby producing elevated serum levels of VEGF in patients. VEGF-mediated endothelial cell dysfunction is an etiological factor of diabetic complications, and serum VEGF levels are significantly higher in DFU patients than in healthy individuals [16]. Stevens et al. [19]

revealed that to a certain extent, these alterations in VEGF levels result from changes in the structure of the VEGF promoter. Nucleotide substitutions in the transcriptional regulatory region of the VEGF gene may alter VEGF protein expression and thus affect the biological functions of VEGF. Therefore, analyzing polymorphisms in the promoter regions of candidate genes such as VEGF can provide strong markers of the genetic basis of complex diseases. Stevens et al. [19] determined that carriage of the -460/+405 polymorphism significantly alters the promoter activity and responsiveness of the VEGF gene, increasing promoter activity by 71% relative to carriage of the wild-type sequence. Amoli et al. [1] examined VEGF gene SNPs at positions -7\*C/T and -2578\*C/A and found that lower frequency of A allele in patients with DFU is conferring a protective effect which might be as a result of increased angiogenesis in patients carrying this allele. In the present study, we investigated the association between VEGF gene promoter polymorphisms and susceptibility to DFU and found that the -460T/C polymorphic locus in the VEGF gene promoter was associated with DFU. Comparison of patients with T2DM alone and the control group showed that the C allele was observed significantly less frequently in the DFU patient group at this locus. Polymorphisms at the -460T/C locus of the VEGF promoter significantly enhances promoter activity [20]. Our results suggested that the C allele at this locus increases angiogenesis, providing protective and preventive effects against DFU.

Local levels of VEGF mRNA decrease after skin tissue injury in DFU patients, leading to deficiencies in angiogenesis at the wound site [10]. As a result, there is an inadequate supply of the required nutrients and oxygen in the wounded region, greatly affecting wound healing. This is an important reason why skin damage repair is difficult for DFU patients. VEGF is a dimeric glycoprotein secreted by a variety of cells. This protein promotes endothelial cell division, induces angiogenesis, enhances microvascular permeability, and improves endothelium-dependent vasodilation. Therefore, regulating VEGF expression levels has important implications for the treatment of DFU. Brem et al. [21] used adenoviral vectors to express VEGF in wound tissues and observed accelerated wound healing in animal models of diabetes. In addition, recombinant VEGF has been used to treat DFU [22].

**Conclusion**

In conclusion, our data indicated that polymorphism at the -460T/C locus of the VEGF promoter is associated with DFU. This finding helps to further clarify DFU pathogenesis from a molecular biological perspective and has major significance for proposing prevention, early diagnosis, and treatment approaches for DFU. However, different races and ethnicities may exhibit different expression of susceptibility genes; thus, broader populations should also be examined. Further studies are required to demonstrate that the results of this study are valid in other populations.

## Acknowledgment

Funding was provided by the Natural Science Foundation of Heilongjiang Province (Grant No. D200970).

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