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Association between *VEGF* genetic variants and diabetic foot ulcer in Chinese Han population

A case–control study

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Abstract

The aim of this study was to investigate the association of vascular endothelial growth factor (VEGF) gene polymorphisms with diabetic foot ulcer (DFU) susceptibility in a Chinese Han population.

Around 88 type 2 diabetes mellitus (T2DM) patients without DFU and 97 T2DM patients with DFU were enrolled in this study. A total of 103 age and gender matched healthy individuals were recruited as healthy control. *VEGF* gene polymorphisms rs699947 and rs13207351 were analyzed by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) method. Odds ratio (OR) with 95% confidence interval (CI) was calculated to assess the association between *VEGF* gene polymorphisms and DFU risk.

The frequency of AA and AC genotypes of rs699947 were lower in DFU patients than that in healthy controls ($P = .020$, $P = .031$), suggesting that AC and AA genotypes were negatively associated with DFU risk originating from healthy individuals (OR = 0.496, 95%CI = 0.274–0.899; OR = 0.130, 95%CI = 0.015–1.112). Significantly decreased trend of rs699947 A allele was observed in DFU patients when compared to the controls ($P = .004$), suggesting A allele was distinctly correlated with decreased DFU risk (OR = 0.490, 95%CI = 0.298–0.804). But no significant differences were detected in rs13207351 genotype and allele distributions between patients and control groups ($P > .05$).

Individuals carrying *VEGF* rs699947 A allele show low susceptibility to DFU in the Chinese Han population.

Abbreviations: 95% CIs = 95% confidence intervals, DFU = diabetic foot ulcer, DM = diabetes mellitus, HIF-1α = hypoxia-inducible factor-1α, HWE = Hardy–Weinberg equilibrium, ORs = odds ratios, PCR-RFLP = polymerase chain reaction restriction fragment length polymorphism, SNPs = single nucleotide polymorphisms, T2DM = type 2 diabetes mellitus, VEGF = vascular endothelial growth factor, VEGFR1 = vascular permeability factor receptors Fit-1, VPF = vascular permeability factor.

Keywords: DFU, polymorphisms, *VEGF*

1. Introduction

Diabetic foot ulcer (DFU) is a particularly debilitating disorder, which is one of the major complications in individuals with diabetes.[1] In addition to diabetes, the development of DFU is often associated with peripheral neuropathy, trauma, and peripheral vascular diseases.[2] DFU has become a significant cause of admission in patients with diabetes.[3] Without prompt treatments, DFU will lead to lower extremity amputation, which may range in severity from the removal of a toe to the amputation of the full lower extremity.[4] The lifetime risk of developing DFU reaches 15% in patients with diabetes, which would result in potential amputation.[5] Among people with diabetes, the annual incidence of DFU has been estimated as 1% to 4%, with the prevalence ranged from 4% to 10%.[6] Consequently, it would be necessary to understand the molecular and genetic basis of DFU.

The pathogensis of DFU is closely associated with the peripheral neuropathy and peripheral vascular disorders. Vascular endothelial growth factor (VEGF), originally known as vascular permeability factor (VPF), is one of the most potent endothelial cell mitogens which plays a crucial role in angiogenesis.[7] VEGF is a 45 kDa homodimetric heparin-bonded glycoprotein that binds with high affinity to the specific receptors Fit-1 (VEGFR1) and kinase insert domain receptor (KDR, VEGFR2). The human *VEGF* gene is located on chromosome 6p21, containing 8 exons and 7 introns.[8] Recent years, several growth factors have been reported to be involved in the development of diabetes mellitus and its complications, in which VEGF has received growing attentions.[9–12] VEGF is a key regulator of integrin-mediated vascular pathways, which participate in the wound healing.[13] To enhance the expression of VEGF may reduce the risk of DFU and promote wound healing[14,15] Furthermore, a major study has suggested a significant association of *VEGF* polymorphisms with DFU susceptibility in an Iranian population.[16] However, the related studies had been rarely reported in Chinese Han population.

Numbers of single nucleotide polymorphisms (SNPs) have been identified in *VEGF* gene. Rs699947 (−2578C/A) and rs13207351 (−125A) polymorphisms were located in the promoter region of *VEGF* gene which may hold the capacity

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to regulate gene expression level. These 2 SNPs have been explored in different diseases, including diabetes and diabetic retinopathy.[17–20] However, the genetic association of these 2 *VEGF* SNPs with DFU risk in Chinese Han population was poorly known. Therefore, we examined the genetic relationship between *VEGF* gene rs699947 (−2578C/A) and rs13207351 (−125A) polymorphisms and DFU susceptibility in present study.

2. Materials and methods

2.1. Subjects

A total of 185 patients with type 2 diabetes mellitus (T2DM) were randomly selected from the diabetes clinic of the First Hospital of Ningbo, who were admitted to the hospital from January 2014 to February 2017. The diagnosis of T2DM was made according to American Diabetes Association Criteria. According to the presence of DFU, the patients were divided into T2DM with DFU (n = 97, male/female = 1.425) and T2DM without DFU (n = 88, male/female = 1.38) groups. The foot ulcer was assessed and graded using Wagner grading system.[21] Besides, 103 healthy controls (60 males and 43 females) who underwent a physical examination during the study time were recruited from the same hospital. All healthy controls had no histories of DFU or related diseases. The three study groups were matched in gender and age.

This case–control study was approved and consented by Ethics committee of the First Hospital of Ningbo. Sample collection was accordance with the ethnic criteria of national human genome research. All participants or their guardians signed informed consents before enrollment. Subjects were Chinese Han population who had no blood relationship with each other.

2.2. Sample collection

Around 5 mL fasting peripheral venous bloods were collected from each participant using the anticoagulative tube with EDTA-disodium salt. Genomic DNA was extracted by TaKaRa Genome DNA Extraction Kit (Dalian Biological Engineering CO., LTD, China). The extracted DNAs were dissolved in sterile and distilled water and then stored at −20°C for standby application.

2.3. Determination of the polymorphisms

Polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) method was applied for the genotyping of *VEGF* gene rs699947 and rs13207351 polymorphisms. Primer sequences for the 2 polymorphisms were designed by Primer Premier 5.0 software, and synthesized by Sangon Biotech (Shanghai, China) (Table 1). PCR procedures consisted of an initial degeneration at 94°C for 5 minutes, followed by 35 cycles of 94°C degeneration for 30 seconds, annealing for 1 minute at different temperatures (53.7°C for rs699947 and 59.0°C for rs13207351), and 72°C extension for 1 minute, and a final 72°C extension for 10 minutes.

PCR products of rs699947 and rs13207351 were digested by specific restriction enzymes, respectively (BgIII for rs699947 and DdeI for rs13207351). Then digested fragments of the 2 SNPs were separated by agarose gel electrophoresis and visualized by UV light. Additionally, PCR products for rs699947 and rs13207351 polymorphisms were randomly selected for genotyping confirmation by direct sequencing method, and the genotyping results were 100% concordant with the PCR-RFLP results.

Table 1

Primer sequences of *VEGF* gene 2 polymorphisms rs699947 and rs13207351.

SNP		Primer sequences	Annealing temperature, °C
rs699947	Sense	5′-CATTCTCAGTCCATGCCTCC- 3′	53.7
	Reverse	5′-GGTTTCTGACCTGGCTATTT- 3′	
rs13207351	Sense	5′-CTGCTCCCTCCTCGCCA-3′	59.0
	Reverse	5′-AGCCTCAGCCCTTCCACA- 3′	

VEGF = vascular endothelial growth factor.

2.4. Statistical analysis

Statistical analysis was performed using the PASW statistics 18.0. Genotype and allele frequencies of *VEGF* gene rs699947 and rs13207351 polymorphisms were calculated by direct counting. Distributions of *VEGF* polymorphisms were checked for the Hardy–Weinberg equilibrium (HWE) via chi-square test. The chi-square test was also used to compare the genotype and allele distributions of *VEGF* gene 2 SNPs between different groups. Association between *VEGF* gene polymorphisms and DFU susceptibility was presented by odds ratios (ORs) and 95% confidence intervals (95% CIs). Statistically significant level was set at $P = .05$.

3. Results

3.1. HWE test

Genotype distributions of *VEGF* gene rs699947 and rs13207351 polymorphisms were all in accordance with HWE test in control group (Table 2, $P > .05$ for both), suggesting the well representativeness of the study samples.

3.2. Genetic association of VEGF gene polymorphisms with DFU

The distribution of *VEGF* gene rs699947 polymorphism was significantly different between DFU patients and control group. Frequencies of AC and AA genotypes were significantly lower in DFU patients than that in healthy controls (28.87% vs 42.72%, $P = .020$; 1.03% vs 5.82%; $P = .048$). However, only rs699947 AC genotype conferred a protective effect for DFU risk which deriving from healthy individuals (OR = 0.496, 95%CI = 0.274–0.899). A allele of rs699947 SNP had significantly reduced trend in DFU patients, when compared with the healthy controls. It suggested that individuals carrying the rs699947 A allele had lower risk to suffer from DFU originating from healthy controls ($P = .004$, OR = 0.490, 95%CI = 0.298–0.804). Additionally, the frequencies of AC and AA genotypes also showed decreased possible to under DFU in T2DM patients, as well as A allele distribution, but all differences did not reach significant level ($P > .05$) (Table 2).

For rs13207351 genotypes and alleles, there was no significant difference discovered between patients and controls ($P > .05$). We speculated that *VEGF* gene rs13207351 polymorphism might have no obvious association with DFU susceptibility in the Chinese Han population (Table 2).

Table 2

Genotype and allele distributions of *VEGF* gene 2 polymorphisms rs699947 and rs13207351 in T2DM subjects without DFU (P) and with DFU (DFU) group, and healthy control group (C).

Genotype/ Allele	*P*, *n*=88 (%)	DFU, *n*=97 (%)	C, n=103 (%)	*P*-value		
				P/C	DFU/C	P/DFU
rs699947						
CC	58(65.91)	68(70.10)	53 (51.46)	–	–	–
AC	27 (30.68)	28 (28.87)	44 (42.72)	.061	.020[a]	.704
AA	3 (3.41)	1 (1.03)	6 (5.82)	.275	.048[b]	.253
C	143 (81.25)	164 (84.54)	150 (72.82)	–		–
A	33 (18.75)	30 (15.46)	56 (27.18)	.052	.004[c]	.401
P_{HWE}			0.422			
rs13207351						
GG	51 (57.95)	61 (62.89)	54 (52.43)	–	–	–
GA	34 (38.64)	33 (34.02)	44 (42.72)	.504	.166	.499
AA	3 (3.41)	3 (3.09)	5 (4.85)	.546	.395	.831
G	136 (77.27)	155 (79.90)	152 (73.79)	–	–	–
A	40 (22.73)	39 (20.10)	54 (26.21)	.430	.148	.538
P_{HWE}			0.290			

DFU=diabetic foot ulcer, HWE=Hardy–Weinberg equilibrium, VEGF=vascular endothelial growth factor.
*AC vs CC, *P*=.020, OR=0.496, 95%CI=0.274–0.899.
†AA vs CC, *P*=.048, OR=0.130, 95%CI=0.015–1.112.
‡*P*=.004, OR=0.490, 95%CI=0.298–0.804.

4. Discussion

DFU is a particularly debilitating disorder caused by multiple factors.[22] Previous studies have regarded DFU as the leading cause of hospitalization for the diabetic patient in the United States and Britain, and this disorder is always correlated with arterial vasculopathy and/or neuropathy. The high glucose condition could damage intracellular calcium channels, thus lead to cardiovascular diseases.[23] The long-term dysregulation of calcium channels may further impair glucose homeostasis and insulin release, thereby aggravating diabetes severity and leading to diabetic complications.[24] Recent study has found that DFU has been a significant cause of morbidity in diabetics. Furthermore, the death rate of DFU cases is increased, due to the elevated burden of cardiovascular diseases.[25] Despite advancing medical technology, the incidence of DFU still exerts increasing trend. DFU has become a major burden on the human health-care system.[26] Accumulating evidences have demonstrated that the development of diabetic neuropathy may be dependent on its complex genetic backgrounds. Furthermore, the prevalence of DFU is distinct in different populations, revealing the potential role of genetic factors in the pathogenesis of DFU.[27] Up to now, several candidate genes have been confirmed to be associated with the onset of DFU, such as the hypoxia-inducible factor-1α (HIF-1α),[28] the heat-shock protein 70 gene.[3] However, the specific etiology of DFU still remained unclear. The related molecular studies are in urgent need.

VEGF acts as a mitogen in vascular endothelial cells.[29] Besides, VEGF can clear the matrix, facilitating migration, and sprouting of endothelial cells, which may induce collagenases and promote angiogenesis.[30] The human *VEGF* gene is located on chromosome 6p21, and multiple polymorphisms have been confirmed for the gene. It had been reported that *VEGF* gene polymorphisms were associated with T2DM, as well as its complications.[31]

In the present study, *VEGF* gene rs699947 and rs13207351 polymorphisms distributions were analyzed in a Chinese Han population. We noted that *VEGF* gene rs699947 polymorphism was associated with DFU susceptibility. Individuals carrying AC genotype showed lower risk to develop DFU deriving from healthy controls, and the ancestral A allele was a protective factor for DFU onset. In the previous studies, rs699947 polymorphism was reported to be associated with several human diseases, such as atherosclerosis, lung cancer, Alzheimer's disease, even the graft survival. In our present study, the difference of rs699947 was seen in both allelic and genotype distributions between T2DM patients with DFU compared to controls. While the ancestral A allele conferred a protective effect for DFU originating from healthy individuals approximately 0.490-fold. Functional studies have shown that patients carrying the A allele showed higher expression of mRNA, besides, same phenomenon was also found in rs699947 GA and AA genotype carriers.[32,33] It seemed that lower frequency of A allele might lead to the increase of angiogenesis, further reduce the morbidity of DFU. In the previous study, rs699947 has been reported to be associated with DFU susceptibility in an Iranian population, which was in accordance with our present results.[16] In addition to rs699947, *VEGF* gene rs13207351 polymorphism was also analyzed, but no significant differences of genotype and allele distributions were found between all patients and control groups. We speculated that rs13207351 might have no obvious association with DFU susceptibility in the Chinese Han population. A major study has suggested that both rs699947 and rs13207351 polymorphisms showed significant association with diabetic retinopathy (DR) in a cohort of Chinese patients with T2DM. Thus other more studies in different populations were needed to confirm our results obtained in this study.

In conclusion, our data suggested a significant association of *VEGF* gene rs699947 polymorphism with the occurrence of DFU. The minor A allele might reduce the susceptibility to DFU, while no significant association was detected for rs13207351 polymorphism. Although we obtained encouraging results, there were still several limitations in the present study. Firstly, the study sample size was not large enough, meanwhile, all the included patients were from the same region that might limit the application range of our results. Secondly, other genetic factors and environmental factors did not take into the analysis, which might lead to imprecision. Finally, the causality and exact

mechanism of *VEGF* polymorphisms in DFU development was still not clear. Therefore, further studies in other larger of different populations should be conducted to replicate and verify our study results. Furthermore, functional study would also be needed to explore the molecular mechanisms underlying the association.

Author contributions

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