



# Relationship between Gene Polymorphism of Vascular Endothelial Growth Factor (VEGF) rs699947 with VEGF and Matrix Metalloproteinase-14 Protein Levels in Patient with Diabetic Foot Ulcer

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## Abstract

**BACKGROUND:** Vascular endothelial growth factor (VEGF) protein levels in diabetes mellitus (DM) patients with ulcerative foot will tend to decrease. Matrix metalloproteinases (MMPs) and their inhibitors have also been identified in regulating capillary tubes formation (morphogenesis) with the collagen matrix, associated with the formation and regression of granulation tissue during the wound healing process.

**AIM:** This study was aimed to determine the relationship between gene polymorphism VEGF rs699947 with VEGF and MMP-14 protein levels in cases of diabetic foot ulcers (DFUs).

**METHODS:** This study was an observational research with cross-sectional comparative study design. The population in this study were type-2 DM patients who met the inclusion criteria. According to the Meggitt-Wagner classification, the study sample was divided into two groups: Type 2 DM group without DFU and type 2 DM group with DFU Grades 1–3.

**RESULTS:** In this study, there were differences in the protein levels of MMP-14 ( $p = 0.039$ ) VEGF ( $p = 0.002$ ) between type-2 DM patients with and without FDU. However, there was no difference in the VEGF gene polymorphism rs699947 between type-2 DM patients with and without FDU ( $p = 0.099$ ). In addition, the results showed that type-2 DM patients with MMP-14 protein levels  $\leq 3.864$  had a 3.6 times greater risk of suffer FDU compared to type-2 DM patients with MMP-14 protein levels  $> 3.864$  but not significant (PR = 3.600 (IK 5 % 1.142–11.346);  $p = 0.052$ ). Meanwhile, type 2 DM patients with VEGF protein levels  $\leq 567.42$  were significantly more at risk of 9048 times to suffer FDU compared to type 2 DM patients with VEGF protein levels  $> 567.42$  (PR = 9.048 (CI 5% 2.571–31.842);  $p = 0.001$ ).

**CONCLUSION:** In type 2 DM patients with FDU, there were lower levels of MMP-14 and VEGF compared to patients without FDU. There is a significant relationship between VEGF protein levels and the incidence of FDU in type 2 DM patients, but there is no relationship between MMP-14 and the gene polymorphism VEGF rs699947 with the incidence of FDU in type 2 DM patients.

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## Introduction

Diabetes mellitus (DM) is a global health problem that affects  $\pm$  411 million people in 2014 [1]. The incidence of DM has doubled since 1980, based on the World Health Organization data [2]. The increasing population of DM patients impacts the incidence of diabetic foot ulcers (DFU) as a chronic complication of DM. The incidence of DFU tends to increase and becomes a considerable burden in the health-care system [3]. As many as, 15–25% of DM patients will experience DFU in their lifetime [4]. The prevalence of DFU in the diabetic population is 4–10% and is more common in older patients. It is estimated that about 5% of all patients with DM present with a history of DFU, while the lifetime risk of DM patients with this complication is 15% [4]. Without prompt treatment, DFU healing is impaired and has the potential to be amputated. This

complication is one of the leading causes of disability and death in diabetic patients [4].

In recent years, growth factors have been reported to be involved in the progression of DM and its complications. Among the various types of growth factors that play a role in developing advanced complications of diabetes, vascular endothelial growth factor (VEGF) has become the current focus [5]. The role of VEGF is as a mitogen for vascular endothelial cells. It induces the process of collagenesis and angiogenesis by clearing the matrix, facilitating migration, and sprouting of endothelial cells [1], [5].

VEGF gene polymorphisms have been reported to have a role in influencing the level of mRNA expression so that this polymorphism can be a potential marker in analyzing the role of genetics in complex diseases. It has been reported that there is a significant relationship between the VEGF 2578\* C/A polymorphism

and the susceptibility of DFU in the Iranian population [5]. A study by Li *et al.* also reported that the VEGF gene polymorphism rs699947 has a significant role in the exposure of DFU in the Han Chinese population [3].

VEGF protein levels in DM patients with complications of DFU will tend to decrease because diabetic fibroblasts cannot increase VEGF production at normal levels in response to hypoxic conditions. Abnormal levels and activity of VEGF cause disruption of the wound healing process of ulcers in the extremities, especially angiogenesis, so that the cell proliferation phase and matrix deposition are also slower [6].

Matrix metalloproteinases (MMPs) and their inhibitors have also been identified in regulating capillary tubes formation (morphogenesis) with the collagen matrix, associated with the formation and regression of granulation tissue during the wound healing process. MMP MT1-MMP (MMP14) is indispensable in forming endothelial cell tubes to spread the collagen matrix. Some evidence has shown that increased hypoxic conditions and impaired cellular response to hypoxia in diabetic patients are critical pathogenic factors in wound healing failure in DFU. Hyperglycemic conditions in DM patients will increase the formation of mitochondrial reactive oxygen species (mtROS) and induce cellular hypoxia through suppression of aquaporin-1 (AQP1) expression. The hypoxic microenvironment will activate target genes that play a role in wound healing and remodeling, including the VEGF gene [7]. This study was aimed to determine the relationship between gene polymorphism VEGF rs699947 with VEGF and MMP-14 protein levels in cases of DFU.

## Methods

This study is an observational study with a cross-sectional design. The research was conducted at outpatient unit and wards of surgery and internal medicine department in three local hospitals. The hospitals are Jambi General Hospital, Bhayangkara Hospital, and Santa Theresia Hospital in Jambi city. The period of study was from December 2019 to December 2021. A total of 52 type-2 DM patients were participated in this study. The patients were divided into two groups, 26 patients with DFU Grades 1–3 according to the Meggitt-Wagner classification and 26 subjects without DFU. The research has been received ethical approval from ethical committee of Faculty of Medicine, Andalas University, Indonesia.

The independent variables were gene polymorphism VEGF rs699947, VEGF protein levels, and MMP-14 protein levels. The dependent variable is type-2 DM patients without and with DFU (based on the Meggitt-Wagner classification). VEGF and MMP-14 protein levels were examined with ELISA kit according to the manufacturer's procedure (Sunlong Biotech, Hangzhou, China). Isolation of the VEGF gene and sequencing of DNA

sequences were carried out using the standard method according to the kit used at the Biomedical Laboratory, Faculty of Medicine, Andalas University.

## Results

In this study, it was found that type-2 DM patients with DFU had a mean age of  $56.5 \pm 6.72$  years with a range of 42–69 years, the average length of suffering from type-2 diabetes was  $8.62 \pm 3.79$  years with a range of 5–20 years old, the majority are women and most of the occupations are traders. Statistical analysis showed that there was no difference in age ( $p = 0.683$ ), duration of DM ( $p = 0.415$ ), gender ( $p = 1.000$ ), and occupation ( $p = 0.761$ ) between type-2 DM with and without DFU (Table 1).

**Table 1: Baseline characteristics of patients**

Characteristics	Total	Group		p
	(n = 52)	DFU (n = 26)	Non-DFU (n = 26)	
Age (years old)				0.683 <sup>a</sup>
Mean $\pm$ SD	57.0 $\pm$ 8.71	56.5 $\pm$ 6.72	57.5 $\pm$ 10.44	
Length of suffering from DM (years)				0.415 <sup>b</sup>
Mean $\pm$ SD	7.96 $\pm$ 3.02	8.62 $\pm$ 3.79	7.31 $\pm$ 1.81	
Gender				1.000 <sup>c</sup>
Male	10 (38.5)	11 (42.3)	21 (40.4)	
Female	16 (51.6)	15 (57.7)	31 (59.6)	
Occupation				0.761 <sup>d</sup>
Civil servants	6 (11.5)	2 (7.7)	4 (15.4)	
Trader	20 (38.5)	10 (38.5)	10 (38.5)	
Housewives	18 (34.6)	9 (34.6)	9 (34.6)	
Farmer	8 (15.4)	5 (19.2)	3 (11.5)	

<sup>a</sup>Independent t-test,  $p < 0.05$ ; <sup>b</sup>Mann-Whitney U-test,  $p < 0.05$ ; <sup>c</sup>Chi-square,  $p < 0.05$ ; <sup>d</sup>Pearson Chi-square,  $p < 0.05$ .

The mean protein level of MMP-14 in type-2 DM patients with DFU was obtained at  $3.822 \pm 0.702$  ng/ml with a range of 2.971–5.558 ng/ml while the mean protein level of VEGF was  $539.02 \pm 74.50$  ng/ml with a range of 429.112–782.591 ng/ml. Statistically, there were differences in the protein levels of MMP-14 ( $p = 0.039$ ) and VEGF ( $p = 0.002$ ) between type-2 DM patients with and without DFU (Table 2).

**Table 2: Laboratory finding of each group**

Laboratory finding	Total	Group		p
	(n = 52)	DFU (n = 26)	Non-DFU (n = 26)	
MMP-14 (ng/ml)				0.039 <sup>a</sup>
Mean $\pm$ SD	4.229 $\pm$ 1.266	3.822 $\pm$ 0.702	4.636 $\pm$ 1.559	
VEGF (ng/ml)				0.002 <sup>b</sup>
Mean $\pm$ SD	599.15 $\pm$ 143.88	539.01 $\pm$ 74.50	659.29 $\pm$ 170.76	
Gene polymorphism VEGF rs699947				0.099 <sup>b</sup>
Yes	6 (13.5)	20 (76.9)	25 (96.2)	
No	45 (86.5)	6 (23.1)	1 (3.8)	

<sup>a</sup>Mann-Whitney,  $p < 0.05$ ; <sup>b</sup>Chi-square,  $p < 0.05$ .

Gene polymorphism VEGF rs699947 in this study occurred in 45 patients (86.5%). In the type-2 DM group with DFU, gene polymorphism VEGF rs699947 occurred in 20 patients (76.9%) while in the type-2 DM group without DFU, gene polymorphism VEGF rs699947 occurred in 25 patients (96.2%). Statistical analysis showed that there was no difference in the gene polymorphism VEGF rs699947 between type-2 DM patients with and without DFU ( $p = 0.099$ ).

By analysis of the ROC curve, the cut-point value of MMP-14 protein levels based on the incidence of DFU in type-2 DM patients was 3.864 (AUC = 0.666 [95% CI 0.519–0.814]). Meanwhile, the cut-point value of VEGF protein levels was based on the incidence of DFU in type-2 DM patients was 567.42 (AUC = 0.754 [CI 95% 0.619–0.890]) (Figure 1).

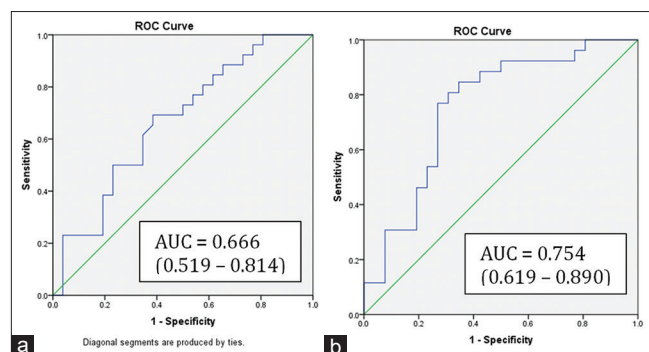


Figure 1: ROC curve for MMP-14 (a) and vascular endothelial growth factor (b) protein levels

Statistical analysis showed that there was a non-significant relationship between MMP-14 protein levels and the incidence of DFU in type-2 DM patients (Table 3). Type-2 DM patients with MMP-14 protein levels  $\leq 3.864$  had a 3.6 times greater risk of suffer DFU than type-2 DM patients with MMP-14 protein levels  $> 3.864$  but not significant (PR = 3.600 [CI 5% 1.142–11.346];  $p = 0.052$ ). However, there was a significant relationship between VEGF protein levels and the incidence of DFU in type 2 DM patients. Type-2 DM patients with VEGF protein levels  $\leq 567.42$  were significantly more at risk of 9,048 times to suffer DFU compared to type 2 DM patients with VEGF protein levels  $> 567.42$  (PR = 9.048 [CI5% 2.571–31.842];  $p = 0.001$ ).

Table 3: Relationship between MMP-14 and VEGF protein levels with the incidence of DFU in type-2 DM patients

Variable	Type-2 DM		PR (IK 95%)	p
	DFU (n = 26)	Non-DFU (n = 26)		
MMP-14				
$\leq 3.864$	18	10	3.600	0.052
$> 3.864$	8	16	(1.14211.346)	
VEGF				
$\leq 567.42$	20	7	9,048	0.001
$> 567.42$	6	19	(2.57131.842)	

Chi-square test,  $p < 0.05$ . VEGF: Vascular endothelial growth factor.

In the group of patients with gene polymorphism VEGF rs699947, 20 of 45 patients (44.4%) had DFU, while in the group of patients without gene polymorphism VEGF rs699947, 6 of 7 patients (85.7%) had DFU. Statistical analysis showed that there was a non-significant relationship between the gene polymorphism VEGF rs699947 and the incidence

Table 4: Relationship between the gene polymorphism VEGF rs699947 genotype and the incidence of DFU in type-2 DM patients

Genotype	Type-2 DM		p value
	DFU (n = 26)	DFU (n = 26)	
Genotype, n (%)			
A	6 (23.1)	1 (3.8)	0.187
AC	7 (26.9)	11 (42.3)	
C	9 (34.6)	11 (42.3)	
CA	4 (15.4)	3 (3.8)	

Chi-square test,  $p < 0.05$ . VEGF: Vascular endothelial growth factor.

of DFU in type-2 DM patients (Table 4). Type 2 DM patients without gene polymorphism VEGF rs699947 had a 7,500 times greater risk of developing DFU than patients with VEGF gene polymorphism rs699947 but not statistically significant (PR = 7.500 (5% CI 0.833–67.494);  $p = 0.099$ ) (Table 5).

Table 5: Relationship between the gene polymorphism VEGF rs699947 genotype and VEGF protein level

Genotype	VEGF protein level		p
	$\leq 567.42$ (n = 27)	$> 567.42$ (n = 25)	
Genotype, n (%)			
A	5 (18.5)	2 (8.0)	0.716
AC	9 (33.3)	9 (36.0)	
C	10 (37.0)	10 (40.0)	
CA	3 (11.1)	4 (16.0)	

Chi-square test,  $p < 0.05$ . VEGF: Vascular endothelial growth factor.

In addition, there was no difference in genotype of the VEGF gene polymorphism rs699947 with the incidence of DFU ( $p = 0.187$ ), with VEGF protein levels ( $p = 0.716$ ), and with MMP-14 protein levels ( $p = 0.541$ ) (Table 6).

Table 6: Relationship between the gene polymorphism VEGF Rs699947 genotype and the incidence of DFU in type-2 DM patients

Genotype	MMP-14 protein level		p
	$\leq 3.864$ (n = 28)	$> 3.864$ (n = 24)	
Genotype, n (%)			
A	4 (14.3)	3 (12.5)	0.541
AC	10 (35.7)	8 (33.3)	
C	12 (42.9)	8 (33.3)	
CA	2 (7.1)	5 (20.8)	

Chi-square test,  $p < 0.05$ . VEGF: Vascular endothelial growth factor.

## Discussion

VEGF was initially described as an endothelial cell-specific mitogen [7]. VEGF is produced by many cell types, including tumor cells, macrophages, platelets, keratinocytes, and renal mesangial cells [8], [9]. VEGF acts as a significant regulator of neovascularization and an essential inducer of vascular permeability. Over the past few years, it has been recognized that VEGF has additional non-vascular functions. VEGF plays a role in normal physiological processes such as bone formation, hematopoiesis, and wound healing [10].

VEGF is a mediator that regulates angiogenesis and is believed to be one of the essential pro-angiogenic mediators during wound healing [11]. VEGF, which is typically expressed at low levels by epidermal keratinocytes, is upregulated in these cells in injured skin. Keratinocytes can produce VEGF at the beginning of the wound healing process, but keratinocytes can also produce VEGF at an advanced stage of wound healing [12]. Hypoxia is one reason why VEGF is increased during wound healing. Oxidants produced in response to injury, such as hydrogen peroxide, and various other mediators produced at the wound site, such as epidermal growth factor, keratinocyte growth factor, altered growth factor, and tumor necrosis factor, also stimulate the production of VEGF by keratinocytes [13], [14].

This study found that the mean VEGF protein level in type-2 DM patients with DFU was significantly lower than type-2 DM patients without DFU ( $539.02 \pm 74.50$  ng/ml vs.  $659.29 \pm 170.76$  ng/ml). These results are similar to the theory that increased VEGF expression can reduce the risk of DFU and promote wound healing. This shows that low VEGF results in diabetic foot ulcers in type-2 DM patients. VEGF protein levels in DM patients with complications of DFU will tend to decrease because diabetic fibroblasts are not able to increase VEGF production at normal levels in response to hypoxic conditions [6]. Abnormal levels and activity of VEGF cause disruption of the wound healing process of ulcers in the extremities, especially angiogenesis so that the cell proliferation phase and matrix deposition are also slower.

In addition, a large study has reported a significant association of VEGF polymorphisms with DFU susceptibility in an Iranian population [3]. Some nucleotide polymorphisms (SNPs) have been identified in the VEGF gene. The rs699947 (-2578C/A) polymorphism is located in the promoter region of the VEGF gene, which may have the capacity to regulate gene expression levels. These SNPs have been explored in various diseases, including diabetic ulcers and diabetic retinopathy [15]. In this study, the VEGF gene polymorphism rs699947 occurred less in patients with DFU than in type-2 DM patients without DFU (76.9% vs. 96.2%).

In this study, the cutoff point for VEGF protein levels based on the incidence of DFU in type 2 DM patients was 567.42 (AUC = 0.754 [95% CI 0.619–0.890]). VEGF protein levels 567.42 were found more in patients with DFU. In addition, there is a significant relationship between VEGF protein levels and the incidence of DFU in type 2 DM patients. VEGF protein levels > 567.42 (PR = 9.048 [CI 5% 2.571–31.842];  $p = 0.001$ ). However, for the gene polymorphism genotype VEGF rs699947, there was no relationship with VEGF protein levels ( $p = 0.716$ ).

VEGF is increased in the pathogenesis of diabetic complications because it stimulates angiogenesis, microvascular hyperpermeability, and endothelial-dependent vasodilation. This has been demonstrated by its changes at local or regional and serum levels in various complications. Endothelial dysfunction as a mechanistic-unifying link in the pathogenesis of diabetic complications is mediated in part by VEGF. VEGF also induces collagenase, which promotes angiogenesis by cleaning the matrix, facilitating migration, and growth of endothelial cells [12].

Another critical process during wound healing is granulation tissue regression, which includes removing new blood vessels, extracellular matrix, and leukocytes [16]. Matrix metalloproteinases (MMPs) play an essential role in their inhibitors in regulating extracellular matrix deposition and degradation in the

wound healing process [17]. The timely expression of MMP at the time of injury is very influential in the success of wound healing. MT-MMP (MMP-14) plays an essential role in epithelial cell proliferation during wound healing by changing the expression of the KGF51 receptor. In addition, the function of MT-MMP accelerates the migration of epithelial cells *in vitro* through the cleavage of syndecan-1 and laminin-332 [18].

In line with the theory, in this study, the mean MMP-14 protein levels in type-2 DM patients with DFU were significantly lower than type-2 DM patients without DFU ( $3.822 \pm 0.702$  ng/ml vs.  $4.636 \pm 1.559$  ng/ml). The cut-point value of MMP-14 protein levels based on the incidence of DFU in type 2 DM patients was 3.864 (AUC = 0.666 [CI 95% 0.519–0.814]). MMP-14 protein levels 3.864 were found to be more in patients with DFU. In addition, there is a significant relationship between MMP-14 protein levels and the incidence of DFU in type 2 DM patients with MMP-14 protein content > 3.864 but not statistically significant (PR = 3.600 [CI5% 1.142–11.346];  $p = 0.052$ ). There was also no difference in the genotype of the gene polymorphism VEGF rs699947 with MMP-14 protein level ( $p = 0.541$ ).

Various physiological disorders cause impaired healing in DFUs, such as impaired cell migration due to inadequate angiogenesis [17]. Matrix metalloproteinases (MMPs) and their inhibitors have been identified in regulating capillary tubes formation (morphogenesis) with the collagen matrix, associated with formation and regression of granulation tissue during the wound healing process. MMP-14 is indispensable in forming endothelial cell tubes for dispersing the collagen matrix.

## Conclusion

It can be concluded that the low levels of MMP-14 and VEGF in type-2 DM patients in this study influenced the occurrence of DFUs in type-2 DM patients.

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