



## Low level of plasma VEGF-A and C allele of -2578\*C/A polymorphism in the VEGF-A gene are risk factors of diabetic foot ulcer in Javanese ethnic

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### ABSTRACT

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Diabetes mellitus (DM) is caused by abnormal insulin secretion, impaired insulin action, or both. Approximately 12-25% of type 2 diabetes mellitus (T2DM) patients will develop diabetic foot ulcers (DFU). Vascular endothelial growth factor (VEGF) is a group of platelet-derived growth factors (PDGF) which have a potential role in angiogenesis. Low levels of VEGF-A can cause insufficient angiogenesis leading to wound healing inhibition. The 2578\*C/A polymorphism of VEGF-A gene has been reported as a candidate marker for the DFU development. However, the variant role in the development of DFU in Javanese ethnic needs to be clarified. This study was conducted to compare VEGF-A levels and the -2578\*C/A polymorphism of the VEGF gene among diabetic patients with and without DFU in Javanese ethnic. In this case-control study, the T2DM individuals with DFU as case group (n=19) and without DFU as control group (n=41) were recruited. The VEGF-A levels were determined by ELISA. The ARMS-PCR technique was applied to investigate the presence of -2578\*C/A polymorphism of the VEGF gene. Data were analyzed with independent t, Mann-Whitney, Chi-square, and Kruskal-Wallis tests with significance level of  $p < 0.05$ . The median of plasma VEGF-A level was significantly different between case and control groups ( $p = 0.001$ ). The genotype frequency of -2578\*C/A polymorphism of VEGF gene was no difference between case and control groups. However, individuals with C allele have a higher risk factor to develop DFU than A allele (CC+CA vs AA;  $p = 0.042$ ; OR=2.5). The plasma VEGF-A levels were lower in T2DM subjects with DFU than those without DFU. In conclusion, individuals with C allele of -2578\*C/A polymorphism of VEGF gene are more susceptible to have DFU than individuals with A allele in Javanese ethnic with T2DM.

### ABSTRAK

Diabetes mellitus disebabkan gangguan sekresi dan aksi insulin atau keduanya. Antara 12-25% pasien diabetes mellitus tipe 2 (DMT2) akan mengalami ulkus kaki diabetik (DFU). *Vascular endothelial growth factor* (VEGF) adalah kelompok *platelet-derived growth factors* (PDGF) yang punya peran potensial dalam angiogenesis. Kadar rendah VEGF-A dapat menyebabkan kurangnya angiogenesis sehingga menghambat proses penyembuhan. Polimorfisme 2578\*C/A gen VEGF telah dilaporkan sebagai calon penanda untuk terjadinya DFU. Namun demikian, peran varian gen dalam berkembangnya DFU pada suku Jawa perlu dibuktikan. Penelitian telah dilakukan untuk membandingkan kadar VEGF-A dan polimorfisme -2578\*C/A gen VEGF pada penderita diabetes suku Jawa dengan dan tanpa DFU. Penelitian kasus control ini melibatkan pasien

**Keywords:**  
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DMT2 dengan DFU sebagai kelompok kasus (n=19) dan tanpa DFU sebagai kontrol (n=41). Kadar VEGF-A ditetapkan dengan metode ELISA. Teknik ARMS-PCR digunakan untuk mendeteksi adanya polimorfisme -2578\*C/A gen VEGF. Data dianalisis menggunakan uji t independent, Mann-Whitney, Chi-square dan Kruskal-Wallis dengan tingkat kemaknaan  $p < 0.05$ . Nilai median kadar VEGF-A plasma berbeda bermakna antara kelompok kasus dan kelompok kontrol ( $p=0.001$ ). Frekuensi genotype polimorfisme -2578\*C/A gen VEGF tidak berbeda bermakna antara kelompok kasus dan kontrol. Namun demikian, pasien yang membawa alel C mempunyai factor risiko lebih tinggi berkembangnya DFU dibandingkan yang membawa alel A (CC+CA vs AA;  $p=0.042$ ; OR=2.5). Kadar VEGF-A plasma pasien DMT2 dengan DFU lebih rendah dibandingkan pasien tanpa DFU. Dapat disimpulkan, pasien yang membawa alel C -2578\*C/A polimorfisme gen VEGF lebih rentan terhadap berkembangnya DFU dibandingkan pasien yang membawa alel A pada suku Jawa penderita DM.

## INTRODUCTION

Diabetic foot ulcer (DFU) is a common infection among patients with diabetes mellitus (DM) due to tissue invasion accompanied by microorganism's proliferation leading to tissue damage with or without an inflammatory response of the host cell.<sup>1</sup> Approximately 12-25% of diabetic patients will develop DFU.<sup>2</sup> While its prevalence is low in Western countries, India is the country with the highest rate of amputation for DFU in Asia. It was reported that 3.6% of DM patients in India developed DFU in 1994.<sup>3</sup> In Indonesia, while the exact number of DFU is unknown, the population of DM patients has been increasing and was ranked 7<sup>th</sup> in the world with approximately 10 million cases in 2015.<sup>4</sup> The diabetes complications such as DFU are also predicted to be higher with the rising of DM's annual prevalence which subsequently reduces the quality of life of patients. Poor glycemic and hygienic control of diabetic patients play an important role in the increasing chance of skin infection and the cell's reduced responsiveness against microorganisms.<sup>5</sup>

Angiogenesis plays an important role in the wound healing process. Vascular endothelial growth factor-A (also referred to as VEGF) is a group of platelet-derived growth factors (PDGF) which has a potential role in angiogenesis.<sup>6</sup> Wilgus *et al.*<sup>7</sup> reported the important role of VEGF-A in wound healing. Increased

levels of VEGF-A are related to the alteration of the scar and the recovery of fetus skin fibrosis, also reduction of scars, and improved collagen quality. Vascular endothelial growth factor improved wound healing in diabetic wounds. The VEGF also induced chemotaxis and angiogenesis. Angiogenesis inhibition would inhibit wound healing.<sup>7</sup>

The -2578\*C/A polymorphism of the VEGF-A gene is one of the polymorphisms located in the 5'untranslated promoter region.<sup>8</sup> Marsh *et al.*<sup>9</sup> reported that this polymorphism reduces mRNA expression, which influences the tissue wound healing process.<sup>10</sup> Amoli *et al.*<sup>11</sup> investigated -2578\*C/A VEGF gene variations and found that this variant was the candidate risk factor of DFU development in an Iranian population. Shahbazi *et al.*<sup>12</sup> reported that -2578\*C/A polymorphism of the VEGF gene leads to diminished VEGF level.

This study was conducted to compare plasma VEGF levels and to investigate the -2578\*C/A polymorphism of the VEGF-A gene among diabetic patients with and without DFU in a Javanese ethnic.

## MATERIALS AND METHODS

### Subjects

It was a case-control study. The subjects were divided into two groups. The case group was T2DM patients with DFU, and the control group was T2DM patients without DFU. Subjects were

recruited from Dr. Sardjito General Hospital, Yogyakarta, Indonesia.

The physical examination was used to determine the DFU status. The Wagner Grading System was performed for foot ulcer assessing, and grading. The anamneses and family pedigree were used to confirm the ethnic status. The Javanese men or women aged 30-65 years old who have been diagnosed with T2DM at least 5 years and DFU were recruited as the case group. The patients with the same previous characteristics but without DFU were included as the control group. The patient informed consent forms were signed by all patients which were included in this study. Five mL of venous blood was collected in EDTA tubes and examined for VEGF-A plasma level and genotyping of VEGF-A gene. Ethical clearance of this study was obtained from the Medical and Health Research Ethic Committee, Faculty of Medicine Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta.

#### **Enzyme-linked immunosorbent assay (ELISA)**

The plasma VEGF-A levels were measured by enzyme-linked immunosorbent assay (ELISA) using Abcam®Kit according to the manufacturer's instruction.

#### **DNA Isolation**

DNA isolations were carried out using

Tianamp blood DNA kit (Tiangen®). The DNA yields were stored at -20°C.

#### **Tetra-primer amplification refractory mutation system-polymerase chain reaction (ARMS-PCR)**

The ARMS-PCR technique described by Amoli *et al.*<sup>11</sup> was performed to determine -2578\*C/A polymorphism of the VEGF-A gene and used five primers (TABLE 1).<sup>11</sup> Three types of master mix were made based on the type of primer; the first reaction of master mix contained generic forward primer and antisense primer C, the second reaction of master mix contained generic forward primer and antisense primer A, and the third reaction of master mix contained the primer of beta actin. Two µL of DNA solution was added to the 4.2 µL of master mix. As much as 13.8 µL nuclease-free water was added to the solution.

Twenty µL of mixtures were amplified on the thermocycler (ESCO®). The hot-start step was carried out for 10 min at 95°C. The following 35 cycles of 1 min for denaturation at 95°C, 1 min for annealing at 60°C, 1 min for elongation at 72°C were performed. Final elongation was carried out for 7 min at 72°C. The amplicons were electrophoresed in 2% of agarose gel and visualized under UV illumination. Genotypes were identified by the presence or absence of the amplified target sequence. The expected PCR product was 223 bp for VEGF-A and 315 bp for beta actin.

TABLE 1. List of primers used in this experiment according to Amoli *et al.*<sup>11</sup>

	Primer Sequence
Generic forward primer	5'-TTAGGACACCATAACCGATGG-3'
C anti- sense primer	5'-TCTGATTATCCACCCAGATCG-3'
A anti- sense primer	5'-TCTGATTATCCACCCAGATCT-3'
Forward (beta actin)	5'-CTTCCTTCCTGGGCATGGAG-3'
Reverse (beta actin)	5'-TGGAGGGGCCGACTCGTCA-3'

### Statistical analysis

Characteristics of the subjects were analyzed by Independent t-test or Mann-Whitney test. Genotype and allele frequencies were analyzed by the Chi-square test. The distribution of plasma VEGF-A levels in individuals with T2DM and without DFU was analyzed by Mann-Whitney test. The value was considered significant if the p-value is less than 0.05.

### RESULTS

#### Clinical characteristic of T2DM patients

In this study, the 60 T2DM patients were divided into two different groups.

The 19 patients with DFU were sub-grouped as the case group, while the 41 patients without DFU (NDFU) were sub-grouped as the control group. TABLE 2 shows data of the clinical characteristics of the patients obtained from medical records. There was no significant difference in clinical characteristics between case and control subjects ( $p > 0.05$ ).

#### Plasma VEGF-A level related to diabetic foot ulcer incidence

The median of VEGF-A levels in the case and control subjects are shown in TABLE 3. The VEGF-A level of case subjects was significantly lower than control subjects ( $p = 0.001$ ).

TABLE 2. Clinical characteristics of patients

Variable	DFU (n=19)	NDFU (n=41)	p
Sex			
• Men	6 (%)	16 (%)	0.581
• Women	13 (%)	25 (%)	
Age (year)	54.8±6.7	55.8±6.2	0.597
BMI (kg/m <sup>2</sup> )	22.9 (16.8-24.9)	22.6 (16.1-24.8)	0.709*
Blood pressure (mmHg)			
• Systole	128.6±20.9	127.4±23.7	0.770#
• Diastole	75.5±14.8	77.7±13.1	0.554
Total cholesterol (mg/dL)	202.8±62.9	202.4±40.4	0.824#
Fasting blood glucose (mg/dL)	164.3±54.6	189.1±78.6	0.271#

Data are reported as mean±SD or median (minimum-maximum); \*Mann-Whitney test; #Independent t-test.

TABLE 3. The VEGF-A level of case (DFU) and control (NDFU) groups

Variable	DFU	Non DFU	p
VEGF-A level(pg/mL)	225.9 (27.2-2102.7)	1367.0 (19.5-4908.7)	0.001*

Data are reported as median (minimum-maximum); \*Mann-Whitney test.

### Genotype and allele frequencies distribution of the -2578\*C/A polymorphism in the VEGF-A gene

Three genotypes were found in the control group (CC, CA, and AA), while two genotypes were found in the case group (CC and CA) (FIGURE 1). The distributions of genotypes and alleles were statistically analyzed using Chi-square test (TABLE

4). Chi-square analysis showed that differences of genotype and allele frequencies of -2578\*C/A VEGF-A in the case and control subjects were not statistically significant ( $p = 0.289$  for the genotype frequencies and  $p = 0.653$  for allele frequencies). But the major allele (C allele) conferred a risk factor for the DFU development ( $OR = 2.5$ ;  $p = 0.042$ ).

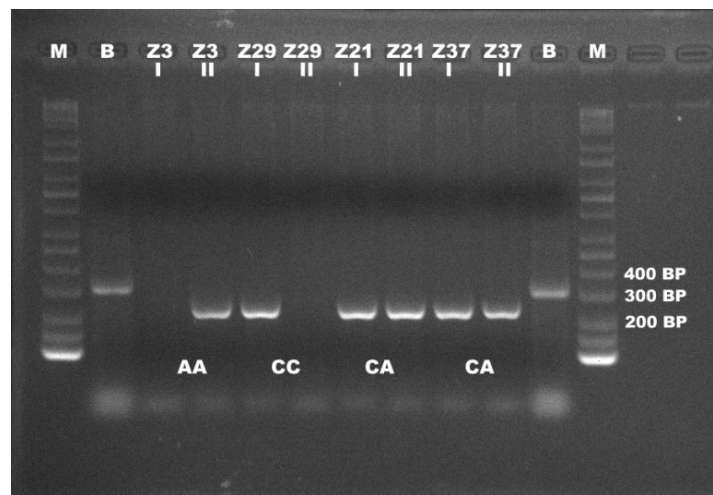


FIGURE 1. Genotype result of -2578 \* C/A VEGF-A gene polymorphism. M = marker. B = beta actin. CC = wild-type. CA = mutant heterozygote. AA = mutant homozygote. Z3=AA, Z29=CC, Z21=CA, Z37=CA.

TABLE 4. Distribution of genotype and allele VEGF-A -2578 \*C/A in case (DFU) and control (Non DFU) subject

Variable	DFU (n=19)	Non DFU (n=41)	p
Genotype			
• AA	0	2 (4.9)	0.289 <sup>*a</sup>
• CA	16 (84.2)	27 (65.9)	
• CC	3 (15.8)	12 (29.3)	
Allele			
• A	16 (42.1)	31 (37.8)	0.653 <sup>*</sup>
• C	22 (57.9)	51 (62.2)	
• CC + CA	19	29	0.042 <sup>*a</sup>
• AA	0	2	

<sup>\*a</sup>Chi square test; <sup>\*</sup> Yates correction

### Association between VEGF-A plasma level with -2578\*C/A VEGF-A polymorphism

The relationship of VEGF-A profile and VEGF-A polymorphisms are shown

in TABLE 5. Results showed that VEGF-A levels in the AA genotype are higher than CC and CA genotypes. However, the VEGF-A levels were not significantly different between that genotype groups after statistical analysis (p= 0.138).

TABLE 5. VEGF-A level in T2DM patients with CC+CA compared to AA genotypes

Variable	T2DM (DFU and Non DFU)		p
	CC + CA	AA	
VEGF-A level (pg/mL)	1120.5 (19.5-4908.7)	2186.8 (1709.9-2663.7)	0.138*

Data are reported as median (minimum-maximum); \*Mann-Whitney test

### DISCUSSION

This study confirmed that a low VEGF-A level leads to poor angiogenesis, and is a risk factor for the development of DFU. The VEGF-A levels of the control group (non-DFU) are significantly higher than the case group (DFU) (p= 0.001). In previous study, the VEGF-A plasma level is associated with the development of diabetic retinopathy and nephropathy. High levels of VEGF-A are a risk factor for diabetic retinopathy. In contrast, our results showed that low levels of VEGF-A act as a risk factor for DFU. Hoong *et al.*<sup>13</sup> examined the VEGF-A levels in T2DM and found that the levels were higher than in healthy subjects. The median VEGF-A level of healthy subjects was 90 (10-230) pg/mL, whereas the median VEGF-A level in the T2DM patients was 180 (120-420) pg/mL.<sup>13</sup> Another study found that VEGF-A levels of diabetic patients were increased relative to healthy controls (928.9±443.2 and 491.7±275.5 pg/mL, respectively).<sup>14</sup> These results support the previous idea that levels of VEGF-A should be maintained within normal ranges to prevent the progression of complications in diabetic patients.

In the aim to investigate the association between VEGF polymorphism and VEGF level, we genotyped subjects for -2578C\*/A polymorphism. The results

showed the presence of three genotypes of CC, CA, and AA. Statistical analysis revealed that the genotype and allele frequencies of -2578\*C/A polymorphisms of the VEGF-A gene are not significantly different between case and control subjects (TABLE 4). In addition, the level of VEGF-A was compared among each genotype to determine the relationship of -2578\*C/A polymorphisms. The VEGF-A levels in AA genotype patients are higher than those of CC and CA genotype patients, but the difference was not significant statistically (p = 0.138). This result is similar to Habboubi *et al.*<sup>15</sup> who also reported that VEGF-A level of CC, CA, and AA genotype carriers were not significantly different from healthy subjects. But this finding is contrary to the research that was reported by Guerzoni *et al.*<sup>16</sup> who found that VEGF-A expression was diminished due to the AA genotype at -2578 VEGF-A. Apparently, the production of VEGF-A is influenced by both genetic and non-genetic determinants.<sup>17,18</sup> Since many factors contribute to the VEGF-A expression, a direct association between -2578\*C/A polymorphism and plasma VEGF-A level in patients with T2DM is still in debate.<sup>19</sup>

However, when the genotypes are divided based on risk alleles, the CA and CC groups are more susceptible to DFU compared to the AA (OR = 2.5; p =

0.042) (TABLE 4). This means that the probability of patients who carry CC and CA genotypes developing DFU is 71.4% higher than the AA genotype. Since the C allele is a common one, many patients with T2DM are actually prone to DFU in the Indonesian Javanese population. The mechanism by which the C allele reduces VEGF-A expression is unknown. The -2578\*C/A polymorphism of the VEGF-A gene is one of the five promoter polymorphisms present in the 5'untranslated region (UTR), which has been identified so far.<sup>8</sup> Several transcription factors bind to this region and polymorphisms located here cause alteration of VEGF-A expression. It is speculated that one C allele is not strong enough to drive low VEGF-A production since the interaction of other transcription factors may occur.<sup>20,21</sup>

High blood glucose levels affect the circulating of angiogenic factors that contribute to the inhibition of wound healing. Natarajan *et al.*<sup>22</sup> reported that hyperglycemia was a major determinant to the elevating of VEGF-A expression. Metabolic balance disorders, such as diabetes can elevate the oxidation of sorbitol to fructose and cause reduction of NAD<sup>+</sup> to NADH. Once the redox balance is disturbed, it would elevate superoxides that induce VEGF production leading to endothelial dysfunction.<sup>23</sup>

Other variables that might be considered in the development of DFU are proinflammatory mediators which increase in patients with T2DM and induce inflammation continuously. Mild inflammation causes impaired cellular defense mechanisms because of hyperglycemia. It has been shown that there is a strong relationship between the development of DFU with proteolytic environment.<sup>24</sup> Impaired wound healing in patients with DM is also caused by a reduction of angiogenesis ability. Research shows that the number of growth factors has decreased in diabetic wounds caused by proinflammatory

mediators.<sup>25</sup> The release of VEGF, PDGF, and TGF- $\beta$  from platelets, monocytes, and fibroblasts induces angiogenesis that is important in the wound healing process.<sup>26</sup> If these growth-factors were reduced, insufficient angiogenesis will occur.

Most diabetic patients undergo peripheral polyneuropathy, as the most common form of diabetic neuropathy.<sup>27</sup> Diabetic neuropathy is another risk factor of developing foot ulcers since patients will have a loss of sensation because of nerve damage.<sup>28</sup> VEGF-A is not the only factor that affects the wound healing of DFU. Pathophysiology of DM and impaired wound healing are very complex. Diabetic patients undergo an abnormal development process of wound healing compared with the normal subjects. It is caused by the association of diabetes and microangiopathy and also their impaired immune system.<sup>29,30</sup> Therefore, research on these topics are very important to understand the pathophysiology of DFU and to overcome this problem in Indonesia. One limitation of this research is the small sample size. We had difficulty recruiting samples because some patients with DFU had comorbid complications with other diseases that affect the angiogenesis process.

## CONCLUSION

The plasma VEGF-A levels are lower in T2DM subjects with DFU than those without DFU. Individuals with C allele of -2578\*C/A polymorphism of the VEGF gene are more susceptible to have DFU than individuals with A allele in Javanese ethnic with T2DM.

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