Association of vascular endothelial growth factor gene +405 C>G and -460 C>T polymorphism with diabetic foot ulcer in Indonesia

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Abstract. Background. The greatest risk factor for Diabetic foot ulcer (DFU) is neuropathy. Vascular endothelial growth factor (VEGF) gene is a gene encodes a protein vascular endothelial growth factor (VEGF), which has a function of angiogenesis and neurogenesis. VEGF plays a role in neuropathy, angiopathy and wound healing in DFU. Methods: Case-control study, case is types 2 DM with DFU and control is type 2 DM without DFU, Polymerase Chain Reaction-Restriction Fragment length polymorphism was done to find genotype polymorphism of VEGF gene. Results: Genotype GG VEGF +405C>G does not have a significant association with DFU in DM patients (GG + CG / CC; OR; 0.52, 95% CI; 0.15 to 1.73 p; 0.289). G allele is proposed as a protective factor in DFU (OR; 0.86, 95% CI; 0.57 to 1.28, and p; 0.456). Genotype TT from VEGF gene -460 C>T; have no significant association with DFU (TT + CT / CC; OR; 0.97, 95% CI; 0.41 to 2.26 and p; 0.942). T allele is predicted as protective factor in DFU (OR; 0.90, 95% CI; 0.59 to 1.37 and p; 0.641). Conclusion: G alleles and T alleles are predicted as a protective factor in DM patients associated with DFU.

1. Introduction
Diabetes mellitus (DM) occurs in the endocrine system, DM experiences by around 171 million people in the world in 2000 and is expected to increase to 366 million in 2030 [1]. According to the International Diabetes Federation (IDF), the number of diabetics in Indonesia reached 8 million people in 2013, this data places Indonesia as the 7th country in the world in population suffering DM after China, India, the United States, Brazil, Russia, and Mexico. The prevalence of DM will continue to increase throughout all of the world, especially at a young and groups of obese people [2–4]. Diabetic Foot Ulcer (DFU) is all ulcer occurs in the foot as a result of diabetes or its complications [5]. DFU is the main cause of DM patients treated at the Hospital. In developing countries, the incidence rate of DM in old age is very high. Every year there are 4 million people with diabetes experiencing DFU. Around the world, at this time there is one case of amputation due to DM every 30 seconds [5–8].
Vascular endothelial growth factor (VEGF) has several roles in the DFU process, ranging from a protective role in the condition of neuropathy with the ability of neurogenesis and angiogenesis, VEGF also has the role of angiogenesis of the microcirculation system in atherosclerosis in peripheral arterial disease (PAD) [3,8]. DFU occurs due to neuropathy, trauma, infection, and ischemia. Neuropathy will cause various changes in the skin, muscles, tendons, and bones and then changes in the pressure distribution of the soles of the feet, further facilitating ulceration. Recurrent trauma, poor limb vascularization conditions, neuropathy and infections affect the process of DFU occurrence and affect wound healing in patients with DM [2,6,9].

Poor blood flow and sustainable of infection make the management of diabetic foot more complexity. This situation provides specificity of DM patients with DFU [8,10]. According to Christian Quantri study in Manchester, VEGF-A gene expression was significantly decreased in diabetics who experienced neuropathy, the results of epidermal VEGF-A biopsy were DM patients compared to controls [11]. Vascular endothelial growth factor (VEGF) also plays an active role in wound healing in DFU sufferers by stimulating the process of angiogenesis, including vasodilation, degradation of basement membranes, endothelial cell migration, and endothelial proliferation, formation of capillary tubules, followed by capillary sprouts (loop formation), and finally the formation of the basement membrane [12]. Until now the pathogenesis of Diabetic Neuropathy (DN) is still unknown well. On histopathological examination, DN patients found atrophy axon abnormalities, damage to nerve fibers, and abnormal nerve regeneration processes. DN pathogenesis is multi-factor involving metabolic components and vascular components [13,14].

At the molecular level, the main factor for DN is due to hyperglycemia, which follows 5 pathways: polyol pathway, glycation end-product pathway, protein kinase C pathway (PKC), poly ADP-ribose polymerase (PARP) pathway, and hexosamine pathway. These five patterns produce oxidative stress. Reactive oxygen species (ROS) induce cell apoptosis and reduce blood flow in nerve tissue. Poly ADP-ribose polymerase (PARP) causes inflammation and nerve dysfunction [15,16]. Decreased blood flow causes microvascular ischemia, resulting in nerve dysfunction. Reactive oxygen species (ROS) causes failure of vasodilation of the epineural blood vessels (vasa nervorum), resulting in ischemia of nerve tissue to and makes diabetic neuropathy (DN) [5,9,10,13].

VEGF encoding genes measuring 14 kb are located on chromosome 6 and have 8 exons and 7 introns (Sfar, et al., 2009 Cit. Amoli et al., 2022). The VEGF gene is located on the chromosome 6p21.3 and consists of 8 exons. Marsh et al. (2000) have conducted a functional study with results indicating that polymorphisms in this gene have an effect on mRNA expression. mRNA expression affects the healing process of wounds that occur in tissues (Rivard et al., 1999). Amoli et al. (2011) reported on the role of genetic factors in a diabetic ulcer [6,17].

VEGF is bound to Flt-1 receptors (VEGFR-1) and KD-R (VEGFR-2), both of which are receptors that have a strong affinity. Flt-1 (VEGFR-1) and KDR (VEGFR-2) are located on the surface of blood vessel endothelial cells 51. In the study of VEGFR-2 receptor mouse (KDR), it was important for differentiation, while VEGFR-1 (Flt-1) was needed for blood vessel formation 40. VEGF-R receptors (VEGFR 1 and 2) are exclusively found in endothelial cells. VEGFR-1 (FL-T) acts to mediate the occurrence of hyper-permeability of blood vessels. While the VEGFR-2 receptor (FLK-1) plays a role in the process of angiogenesis [18].

Vascular endothelial growth factor (VEGF) also induces the formation of collagen and the process of angiogenesis by cleansing the matrix, facilitating the migration process and the growth of endothelial cells [11,17,19]. In research conducted by Allan H. Ropper, et al. In 2009, in the Boston-University Neurology department, USA, it was concluded that the administration of intramuscular VEGF plasmid gene transfers symptomatic symptoms of diabetic neuropathic patients (-1.2 ± 0.5 vs - 0.9 ± 0.5; p <0.01), in the study provide evidence that intramuscular VEGF gene therapy may improve symptoms of diabetic polyneuropathy [20].

2. Methods
The method of this study was a descriptive analytic study polymorphism of VEGF gene at positions +405 C>G and -460 T>C in Diabetic Type 2 with or without DFU Patients at Cipto Mangunkusumo National Hospital Jakarta.
This study was conducted at the Vascular and Endovascular Division Department of Surgery, Cipto Mangunkusumo hospital Jakarta in collaboration with the laboratory of Microbiology and Biomolecular Medical Faculty University of Indonesia and supported any division of Endocrinology Department of Internal Medicine faculty of medicine University of Indonesia Cipto Mangunkusumo hospital Jakarta in September until December 2016.

Table 1. Characteristic of type 2 diabetes mellitus patients with DFU and without DFU at Cipto Mangunkusumo National Hospital from September to December 2016.

| Variable               | DFU (+) | DFU (-) | OR     | 95% CI | p       |
|------------------------|---------|---------|--------|--------|---------|
|                        | n=96    | n=101   |        |        |         |
| Age (year)             |         |         |        |        |         |
| ≥ 70                   | 8       | 6       | 1.26   | 0.37 – 4.33 | 0.707* |
| 60 – 69                | 29      | 23      | 1.18   | 0.52 – 2.75 | 0.671* |
| 50 – 59                | 39      | 53      | 0.69   | 0.33 – 1.48 | 0.351* |
| < 50                   | 20      | 19      | 1.88   | Ref    |         |
| Sex                    |         |         |        |        |         |
| male                   | 48      | 49      | 1.06   | 0.60 – 1.85 | 0.835cs |
| female                 | 48      | 52      | 51.5   | Ref    |         |
| Education              |         |         |        |        |         |
| Primary                | 16      | 15      | 4.00   | 1.39 – 11.44 | 0.010* |
| Junior High            | 34      | 13      | 9.80   | 3.57 – 26.88 | <0.001* |
| Senior High            | 38      | 43      | 3.31   | 1.35 – 8.10 | 0.009* |
| University             | 8       | 30      | 29.7   | Ref    |         |
| Salary                 |         |         |        |        |         |
| < 3,000,000            | 72      | 64      | 1.73   | 0.93 – 3.21 | 0.078* |
| > 3,000,000            | 24      | 37      | 39.7   | Ref    |         |
| Duration of DM         |         |         |        |        |         |
| ≥ 10                   | 52      | 46      | 1.52   | 0.75 – 3.07 | 0.237* |
| 5 – 9                  | 24      | 28      | 1.15   | 0.52 – 2.56 | 0.719* |
| < 5                    | 20      | 27      | 26.7   | Ref    |         |
| BMI                    |         |         |        |        |         |
| Overweight             | 18      | 21      | 0.98   | 0.41 – 2.35 | 0.974* |
| Normoweight            | 58      | 57      | 1.17   | 0.58 – 2.36 | 0.661* |
| Underweight            | 20      | 23      | 22.8   | Ref    |         |
| Neuropathy             |         |         |        |        |         |
| Yes                    | 90      | 62      | 61.4   | 9.43   | 3.76 – 23.63 | <0.001cs |
| No                     | 6       | 39      | 38.6   | Ref    |         |
| Rest pain              |         |         |        |        |         |
| Yes                    | 33      | 4       | 4.0    | 12.70  | 4.29 – 37.59 | <0.001cs |
| No                     | 63      | 97      | 96.0   | Ref    |         |
| Smoking                |         |         |        |        |         |
| Yes                    | 55      | 31      | 30.7   | 3.02   | 1.68 – 5.43 | <0.001cs |
| No                     | 41      | 70      | 69.3   | Ref    |         |
| Hypertension           |         |         |        |        |         |
| Yes                    | 58      | 37      | 36.6   | 2.64   | 1.48 – 4.69 | 0.001cs |
| No                     | 38      | 64      | 63.4   | Ref    |         |
| PAD                    |         |         |        |        |         |
| Yes                    | 54      | 11      | 10.9   | 10.51  | 4.99 – 22.15 | <0.001cs |
| No                     | 42      | 90      | 89.1   | Ref    |         |

The population is the population of Jakarta population type 2 DM patients who come for medication to RSCM. Samples are taken with the technique of consecutive sampling. The Data was analyzed using univariate analysis to view the genotype and distribution of alleles that are experiencing polymorphism by DFU and non-DFU patients.

We used primers of VEGF +405 C>G reverses sequence 5' CGA CGG CTT GGG GAG ATT GC 3' and forward sequence of DNA 5' GGG CGG TGT CTG TCT TGC TG 3' and we used BsmF1 as
enzyme restriction, PCR cycle 95.3’; 95,30’; 72,30’; 72,7’ and electroforesis using gel agarose etidium bromide 2%, Ultra violet visualisation genotype CC; 170bp and 103 bp, GG 273bp, and CG 273 bp, 170 bp and 103 bp [6]. For genotyping VEGF -406 T>C we use primers reverse 5’ TGG CCT TCT CCC CGC TCC GAC 3’, and forward 5’ CCT CTT TAG CCA GAG CCG GGG 3’. Bsa H1 used as restriction enzyme, with PCR cycle 95.3’; 95,30’; 66,30’; 72,30’; 72,7’ [6].

3. Results
The average age of this study was 50 to 59 years old (40% to 50%) and from the data we found that more older patients, there were more risk to become DFU (OR= 0.6 to 1.2). There was no significant difference in DFU between female and male (p=0.835). There was no significant difference in DFU for duartion of DM (p=0.23), sallary (p=0.078) and BMI (p=0.974). There were significant differences risk for DFU between smoking (p=<0.001), neurophy (p=<0.001), rest pain (p=<0.001), PAD (p=0.001), and hypetention (p=<0.001) as shown in table 1.

Table 2. Distribution of etiology DFU in type 2 diabetes mellitus patients in Cipto Mangunkusumo national hospital from in 2016.

| Etiology     | Frekuensi | %    | Kumulatif |
|--------------|-----------|------|-----------|
| Neuropaty    | 50        | 50.5 | 50.5      |
| Combine      | 34        | 34.3 | 84.8      |
| Angiopaty    | 15        | 15.2 | 100       |
| Total        | 99        | 100  |           |

Table 3. Bivariate analysis of VEGF gene polymorphisms in DFU patients at Cipto Mangunkusumo national hospital period of september to december 2016 (n = 197).

| Gen        | Polimorfisme | DFU (+) | DFU (-) | OR    | 95% CI | p    |
|------------|--------------|---------|---------|-------|--------|------|
|            | n=96         | n=101   |         |       |        |      |
| VEGF +405C>G | GG          | 18      | 23      | 0.52  | 0.15 – 1.73 | 0.289 |
|            | CG          | 69      | 72      | 0.63  | 0.21 – 1.89 | 0.418 |
|            | CC          | 9       | 6       | Ref   |        |      |
|            | G           | 105     | 118     | 0.86  | 0.57-1.28 | 0.456 |
|            | C           | 87      | 84      | Ref   |        |      |
| VEGF -460C>T | TT         | 42      | 50      | 0.96  | 0.41 – 2.26 | 0.942 |
|            | CT          | 41      | 36      | 1.31  | 0.55 – 3.12 | 0.537 |
|            | CC          | 13      | 15      | Ref   |        |      |
|            | T           | 125     | 136     | 0.90  | 0.59-1.37 | 0.641 |
|            | C           | 67      | 66      | Ref   |        |      |

In this study we found the distribution of etiology for DFU patients in Cipto Mangunkusumo National Hospital Jakarta were neuropathy 50(50.5%), combine neuroangiopathy 34(34.3%) and angioepathy 15(15.2%) (table 2).

From analysis distribution of genotyping we found that wild type CC genotype of VEGF gene +405 was 7.6%, mutant type CG heterozygote was 71.6% and GG mutant genotype homozygote was 20.8%, the cumulative mutant genotype was 92.4%, there were an increasing in the number of G allele as mutant alleles of 56.6% (table 3).

We also found wild type of VEGF gene -460 which CC genotype were 14.2%, mutant heterozygote CT was 39.1% and TT mutant homozygote was 46.7%. The distribution of the T allele as the VEGF -460 C>T mutant allele was 66.2%. VEGF +405C>G genotype; GG / CC OR; 0.52, 95% CI; 0.15-1.73 and p; 0.289 and CG / CC OR; 0.63, 95% CI 0.21-1.89 and p; 0.418. Results of G allele analysis; OR; 0.86, 95% CI 0.57-1.28 and p; 0.456. VEGF Genotype -460 C>T; TT / CC obtained OR; 0.96,
95% CI; 0.41-2.26 and p; 0.942 and CT / CC obtained OR results; 1.31, 95% CI 0.59-1.37 and p; 0.641. (Table 3).

In this study, DFU scoring was conducted using PEDIS Score which was divided into <9 and > 9 classifications. On VEGF + 405 C> G and VEGF -460 C> T, the results OR 0.54 95% CI 0.1-2.7 and p 0.45 and OR 0.7, 95% CI 0.22 -2.68 with a value of p = 0.68. There was no significant relationship between the degree of Pedis Score with VEGF gene polymorphism (table 4).

**Table 4.** Association of Gene Polymorphism and PEDIS Score in Type 2 DM patients at Cipto Mangunkusumo in 2016 (n = 96)

| Gen Polimorfisme | PEDIS Score <9 | PEDIS Score 9-20 | OR | 95% CI | p |
|------------------|----------------|------------------|----|--------|---|
| VEGF +405C>G     | n | % | n | % | 0.543 | 0,1-2,7 | 0,45 |
| GG               | 9 | 0,09 | 9 | 0,09 |        |        |      |
| CG               | 21 | 0,22 | 48 | 0,50 |        |        |      |
| CC               | 2 | 0,21 | 7 | 0,73 |        |        |      |
| G                | 39 | 0,20 | 66 | 0,34 | 0,68 | 0,3-1,2 | 0,21 |
| C                | 25 | 0,13 | 62 | 0,32 |        |        |      |
| VEGF -460C>T    | TT | 13 | 0,14 | 29 | 0,30 | 0,7 | 0,22-2,68 | 0,68 |
| CT               | 15 | 0,16 | 26 | 0,27 |        |        |      |
| CC               | 4 | 0,041 | 9 | 0,09 |        |        |      |
| T                | 41 | 0,21 | 84 | 0,44 | 0,72 | 0,37-1,37 | 0,32 |
| C                | 23 | 0,12 | 34 | 0,18 |        |        |      |

**4. Discussions**

Foot ulcers is a disabling complication and not uncommon among people with diabetes mellitus. The disability and possible progression to the loss (amputation) of digits and limbs make it a serious issue. This study attempted to examine the risk factors for foot ulceration using a case control design. Systematic assessments done routinely in the special clinic and the computerization of the data were the strengths of the study. Assessment of arterial pulses using Doppler and biothesiometer were not practical and cost effective in secondary care clinical practice and hence were not used in this study. However, assessment using Doppler often give a misleading ankle/brachial index (ABI) in patients with diabetes due to arterial calcification. Foot pulses were used in the clinical assessment, and their absence is usually associated with an ABI of <0.76.

Diabetic patients who came to RSCM who were willing to participate in the study consisted of DM patients with DFU 49% and DM without DFU 51%. Men 49.2% and women 50.8%, this is the same as IDF data that there is no significant difference between gender in the prevalence of DM. 2 According to the IDF almost 50% of all people who suffer from diabetes are in the range of 40 to 59 years and the tendency for DM at a young age to continue to increase due to dietary factors and lack of exercise. In this study, the highest age group was 50-59 years around 46.7%. Based on the average age distribution of 56.33 years.[15]

More than 80% of DM sufferers live in poverty and most in developing countries whose economies are bad, and estimated the number will continue to increase up to 86% in 20352, education level still looked a low educated 49.6% and low monthly income is still below the Jakarta Minimum Regional Wage (UMR) of 3 million IDR 69%. In this study, the average duration of illness was 10.36 years. This is the same as that produced by Mahsa M. Amoli, et al in 2011 at the University of Tehran that the duration of DM illness will increase the occurrence of DFU (p < 0.001, 95% CI 6.82–9.65)1. Neuropathy is a major factor in diabetic ulcers in patients with diabetes, from all samples found there were around 77.2% and more in patients with diabetes with DFU 93.8% compared to patients without DFU 61.4%. Smoking and hypertension are risk factors for vascular disorders and will be fatal in DM patients who experience DFU, from this study it was found that in the DM patients group with DFU 57.3% smokers and 60.4% with a history of hypertension.[21]
Peripheral arterial disease is not always a major risk factor for DFU, but plays a significant role in the possibility of amputation. At the time of diagnosis DM was diagnosed 4% of patients with PAD but after 20 years suffering from diabetes, 45% of these patients will suffer from PAD [7]. Clinical examination by examining the Ankle Brachial Index (ABI) ABI value less than 0.9 was diagnosed as Arterial Disease Peripheral (PAD). In this study, there were approximately 34% of DM patients who came to the RSCM suffering from PAD.

Based on the distribution of DM patients with DFU and without DFU, DM patients with DFU 56.2% suffered from PAD while DM patients without DFU were 10.9% suffering from PAD. Distribution of ulcer causes based on historical results and clinical examination of patients obtained results; neuropathy 50.5%, neuro ischemic 34.3% and angioischemic 15.2%. This result is similar to the result from other study from D Armstrong et Al 2011 said that diabetic foot ulcers can be categorized as purely neuro-pathic, purely ischemic, or a combination of the two, namely, neuroischemic. The estimated current prevalence of each is 35%, 15%, and 50%, respectively [22].

Based on an analysis conducted at the Diabetic Foot Clinic, King’s College Hospital in London, there is some preliminary evidence that the prevalence of neuroischemic ulcers has been rising since the 1990s from approximately one-third of patients to over 50%, therefore becoming the most common etiology of DFUs [22].

Genotype distribution of VEGF gene polymorphism + 405 C> G. Wild-type CC genotype was found at 7.6%, CG mutant heterozygote 71.6% and GG 20.8% mutant homozygote cumulatively obtained 92.4% mutant genotype. Obtained an increase in the number of G alleles as a mutant allele of 223 (56.6%). Genotype distribution of VEGF gene polymorphism -460 C> T; wild-type TT 46.7%, mutant heterozygote CT 39.1% and mutant homozygote CC 14.2%. Distribution of T alleles as mutant alleles in VEGF gene polymorphism -460 T> C T 261 (66.2%).

Results of bivariate genotype analysis of VEGF +405C→G; GG + CG / CC obtained OR; 0.52, 95% CI; 0.15-1.73 and p; 0.289 and CG / CC obtained OR results; 0.63, 95% CI 0.21-1.89 and p; 0.418. Results of G allele analysis; OR; 0.86, 95% CI; 0.57-1.28 and p; 0.456. Results of VEGF -460 C> T bivariate genotype analysis; TT + CT / CC obtained OR; 0.97, 95% CI; 0.41-2.26 and p; 0.942 and CT / CC obtained OR results; 1.31, 95% CI 0.55-3.12 and p; 0.537. Results of T allele analysis; OR; 0.90, 95% CI; 0.59-1.37 and p; 0.641. VEGF polymorphism does not have a significant relationship with DFU disease in DM patients at the RSCM but G and T allele indicate as a protective factor in DFU not as a possible risk factor because of the role of VEGF angiogenesis in DFU. This is in accordance with the study of Mahsa M Amoli, et al in 2013 in Tehran and the study of Wang Hay Yang, et al in 2012 in China [6,23,24]. Our data indicated that polymorphism at the -460T/C locus of the VEGF promotor 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 [9].

5. Conclusions
There was no significant relationship between VEGF +405 C>G and VEGF-460 C>T genes polymorphism with DFU in patients with DM. G and T alleles has as a potential protective factor against the occurrence of DFU in patients with DM in Indonesia.

Our data suggest that VEGF gene polymorphism is a potential candidate gene in the process of DFU in DM patients. We found preliminary evidence regarding the association between VEGF polymorphisms and DFU in diabetic patients. We need a further study with many more samples to confirm the result obtained in this study in different populations.

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