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Vascular Endothelial Growth Factor 936 C/T Gene Polymorphism in Indonesian Subjects with Diabetic Polyneuropathy

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Keywords: *VEGF +936 C/T* polymorphism; VEGF-A level; Diabetic polyneuropathy

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Abstract

AIM: This study aimed to confirm the role of f *VEGF* gene *936 C/T* polymorphism and Diabetic Polyneuropathy (DPN) in the Indonesian population as well as to investigate its relationship with VEGF-A level and the role of vascular risk factors.

MATERIAL AND METHODS: This was a cross-sectional study involving 152 subjects. Clinical symptoms and signs of DPN were examined using DNE and DNS scoring followed by nerve conduction study. All subjects underwent anthropometric, clinical examination and laboratory procedures to obtain body mass index, HbA1C level, lipid profile, Polymorphism of *+936 C/T VEGF* gene (PCR-RFLP technique), and VEGF-A plasma level (ELISA). Statistical analysis using a t-test or Mann-Whitney was performed to assess continuous data and Chi-square for categorical data. Multivariate logistic regressions were also performed to determine the relationship between independent variables and DPN.

RESULTS: Sixty-nine (45.4%) fulfilled the diagnostic criteria of DPN. There was a significant association between *CT + TT* genotype and DPN (OR 0.35 95%CI 0.16-0.79 p = 0.01). Multivariate logistic regression showed that plasma VEGF-A level (OR = 1.003; 95% CI = 1.000-1.007; p = 0.03), diabetes duration (OR = 1.108; 95% CI = 1.045-1.175; p = 0.001), and *CT+TT* genotype (OR = 0.347; 95%CI = 0.148-0.817; p = 0.013) were associated with DPN. Sub-group analysis on subjects with HbA1C level ≥7% showed that VEGF-A (OR = 1.011; 95%CI = (1.004-1.017; p = 0.03), diabetes duration (OR = 1.245; 95% CI = 1.117-1.388; p < 0.001), *CT + TT* genotype (OR = 0.259; 95%CI = 0.074-0.911p = 0.035), with an adition of HDL (OR = 0.916; 95% CI = 0.857-0.978; p = 0.009) were significant predictors of DPN while LDL (OR = 1.017; 95% CI = 1.000-1.035; p = 0.053) acted as modifying factor.

CONCLUSION: It appeared that *CT + TT* genotype of *VEGF +936* gene might act as a protecting factor for DPN while VEGF-A, diabetes duration, HDL, and LDL acted as risk factors especially on subjects with HbA1C level ≥ 7.

Introduction

Diabetic polyneuropathy (DPN) is the most common complication of diabetes mellitus affecting the peripheral nervous system. It is associated with high morbidity and mortality rate and contributes to a decrease in quality of life [1] [2]. The global prevalence of DPN is 10% in the first year after the diagnosis and increases to approximately 50% after diagnosed with diabetes mellitus for 25 years [1] [3]. According to a study performed by Soewondo et al., (2010) [4], it is predicted that around 63.5% of Indonesian diabetic patients treated in tertiary hospitals met the clinical diagnostic criteria of DPN. Data about risk factors associated with this fact is not well documented. Understanding this fact is crucial in the context of early detection and prevention.

Growth factor imbalance as well as high blood glucose level and oxidative stress are believed to have a role in the pathogenesis of DPN as found in animal models. Of all those growth factors, special attention should be paid to the circulating Vascular Endothelial Growth Factor-A (VEGF-A). It is predicted of having a potentially neuroprotective capacity, but its

role is highly influenced by glucose level and oxidative stress. A previous study by Deguchi et al. (2009) found that VEGF level increased in the active phase of DPN but decreased in the advance phase [5]. This fact raised some doubt whether VEGF has a potentially beneficial role, or in reverse, is involved in the pathogenesis of DPN.

It is believed that the genetic factor plays an important role in the development of DPN. Of all genetic variants associated with circulating VEGF level, the polymorphism of *936 3'UTR* of *VEGF* gene has been studied and has shown various results. Zhang *et al.,* (2014) found that the *CC* genotype was associated with an increased risk of developing DPN while *CT* and *TT* genotype was associated with a decreased risk. *CC* genotype also associated with a higher level of serum VEGF-A level [6]. On the other hand, Kim et al. (2009) found that there was no relationship between *936 C/T VEGF* gene polymorphism with DPN although TT genotype was associated with higher plasma VEGF-A level [7].

Vascular risk factors such as high levels of HbA1C, total cholesterol, triglyceride and low level of HDL as well as obesity have also been associated with the risk of developing DPN [6] [8]. These suggest that DPN is a multifactorial condition, a combination of various factors such as metabolic, vascular, and genetics.

Methods

This was a cross-sectional study evaluating the relationship between polymorphism of *VEGF* gene *936 C/T 3'UTR* with DPN. It was conducted in Atma Jaya Academic Hospital, Jakarta between September 2017 and March 2018. Participants were recruited consecutively and had undergone general and neurological clinical examinations, blood tests, and nerve conduction studies. Body mass index, blood pressure, results of the genetic test to evaluate polymorphism of *VEGF* gene *936 C/T 3'UTR*, plasma VEGF-A level, HbA1C, lipid profile, and results of nerve conduction studies were documented. DNA analyses were performed in the Biomedical Laboratory of the School of Medicine Unika Atma Jaya Jakarta and the Laboratory of Medical Parasitology, Faculty of Medicine, Public Health, and Nursing Universitas Gadjah Mada Yogyakarta. The plasma VEGF-A level analysis was performed in Prodia Lab Jakarta, and other laboratory tests were done in the Clinical Pathology Department, School of Medicine, Unika Atma Jaya and Atma Jaya Academic Hospital. Nerve Conduction Studies (NCS) were performed in the Diagnostic Installation of Atma Jaya Academic Hospital.

The inclusion criteria of the subjects were (1) adults diagnosed with type-2 diabetes mellitus aged < 67 years, (2) willing to participate in the study and signed informed consent. The exclusion criteria were having (1) a diagnosis of chronic kidney disease and/or on routine hemodialysis, (2) a history of cancer or chemotherapy, (3) an acute exacerbation of chronic osteoarthritis, (3) peripheral arterial disease-defined by a manual ankle-brachial index of > 0.8, (4) acute stroke or myocardial infarction; (5) arrhythmias and or on cardiac pacemaker, (6) severe polyneuropathy (unrecordable NCS) or diabetic ulcer and/or history of diabetic ulcer, (7) a history of erectile dysfunction, constipation, and chronic diarrhea.

This study was approved by the Medical and Health Research Ethics Committee (MHREC), Faculty of Medicine, Public Health, and Nursing Universitas Gadjah Mada.

Diagnosis of DPN was established when the respondent had a positive result for at least one of the diagnostic evaluation. Diagnostic evaluation in this research was performed using (1) Diabetic Neuropathy Symptom (DNS) & Diabetic Neuropathy Examination (DNE) scoring and (2) NCS. DPN is considered positive if DNS score ≥ 1 and DNE score > 3 while findings from NCS suitable to PDN in this research is (1) ≥ 1 abnormality on ≥ 2 separate nerves or (2) ≥ 1 abnormality on at least 2 nerves which must include the sural nerve [9] [10] [11]. General and neurological examinations as well as DNS and DNE scoring were performed by one specifically-trained physician and conducted before the NCS examination. NCS examinations were performed by a neurologist trained in EMG and Evoked Potentials.

The HbA1C examination was performed using Boronat affinity binding method. Whole blood samples were mixed with reagents on Nycocard kit and read using a Nycocard™ Reader. Total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), and triglyceride levels were assessed using the Insert kit from Labtest on an automated biochemical analyser.

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

DNA isolation from venous EDTA blood was performed using *Wizard® Genomic DNA Purification Kit* (Promega) product code A1120. The sequence for upstream and downstream primers were [12]:

- *forward*: 5'-AAG GAA GAG GAG ACT CTG CGC3.'
- *reverse*: 5'- TAT GTG GGT GGG TGT GTC TAC AGG-3.'

DNA amplification using PCR technique was performed using the kit Go Taq® Green Master Mix (Promega). PCR product was then digested using NlaIII enzyme with CutSmart^{R} buffer (New England Biolabs). Digested product and marker were analysed in 2% agarose gel using FloroSafe staining. After the electrophoresis process (110 V for 30 minutes), the result was then photographed and analysed. Digested products were (Figure 1):

1) *CC* genotype: 1 band with product length 198 bp

2) *CT* genotype: 3 bands with product length198 bp, 114 bp, and 84 bp

3) *TT* genotype: 2 bands with product length 114 bp, dan 84 bp.

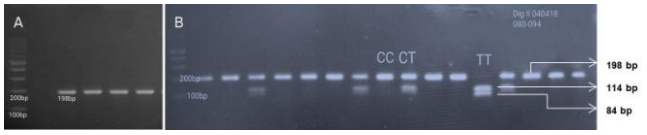

Figure 1: A) Electrophoresis of PCR product showing 1 band (198bp); B) Electrophoresis of Digested PCR Product. CC genotype consists of 1 band (198 bp), CT genotype consists of 3 bands (198 bp, 114 bp, and 84 bp) and TT genotype consists of 2 bands (114 bp and 84 bp). Marker 100 bp on lane 1

Plasma VEGF-A level was detected using ELISA method using ab119566-VEGFA Human ELISA kit from Abcam. Plasma was obtained from centrifuged venous EDTA blood (1500 rpm in 5 minutes) and stored in a refrigerator (-70°C) before the procedure. All steps on the ELISA procedure was conducted according to the protocol written on the kit.

All data were analysed using SPSS version 22. Association between polymorphism of *VEGF* gene *936 C/T* and DPN was determined using Chi-square test. Association between numerical variables with genotype and DPN were evaluated using t-test if the data showed normal distribution and Mann-Whitney U test if not. Logistic regression analysis was then performed to evaluate the relationship between DPN and predictive factors, including genotype and vascular risk factors.

Results

There were 152 patients eligible as study participants. Ninety-eight participants (64.55%) were women, and 54 (35.5%) were men. More than half of the participants were 51-60 years (55.9%). Most of the participants (63.2%) were diagnosed with type-2 diabetes for more than 5 years. Of all participants, 45.4% fulfilled the diagnosis of DPN according to the protocol of this study. Majority of the respondents had HbA1C level ≥ 7% (65.1%). Hypertension was found in 88 (57.9%) of subjects, and 33 (21.7%) were obese. Fifty-two subjects (34.2%) had a cholesterol level of > 200 mg/dl, 108 (71.1%) had an LDL level of > 100 mg/dL, 95 (62.5%) had an HDL level < 40 mg/dL, and 100 (65.8%) had a triglyceride level > 150 mg/dL (Table 1).

Table 1: Baseline characteristics of study subjects

Characteristic	n	%
Sex		
Men	54	35.5
Women	98	64.5
Age (years)		
<40	7	4.6
41-50	26	26.0
51-60	85	55.9
>60	34	22.4
Duration of diabetes diagnosis (years)		
≤ 5	96	63.2
>5	56	36.8
DPN Status		
DPN (-)	83	54.6
DPN (+)	69	45.4
HbA1C (%)		
< 7	53	34.9
≥ 7	99	65.1
Hypertension		
No	88	57.9
Yes	64	42.1
Body Mass Index (kg/m^2)		
< 30, n= 119 (78.3%)	33	78.3
≥ 30, n= 33 (21.7%)	119	21.7
Total cholesterol (mg/dL)		
< 200	100	65.8
≥ 200	52	34.2
LDL (mg/dL)		
< 100	44	28.9
≥ 100	108	71.1
HDL (mg/dL)		
< 40	95	62.5
≥ 40	57	37.5
Triglyceride (mg/dL)		
< 150	52	34.2
≥ 150	100	65.8
Genotype Frequency		
CC	115	75.6
CT	34	22.4
TT	3	2

The frequency of *CC* genotype was 75.6%, *CT* genotype 22.4% and *TT* genotype 2%. Genotype frequency fulfilled Hardy-Weinberg Equillibrium ($\chi 2$ = 0.07; p = 0.787). There was a significant relation between *CT+TT* of *VEGF +936* genotype and diabetic neuropathy (OR 0.35; 95%CI 0.16-0.79; p = 0.01) (Table 2).

Table 2: The relationship between genotype of VEGF gene 936 C/T polymorphism and DPN

		DPN		OR (95% CI)	P
		DPN (-) n (%)	DPN (-) n (%)		
Genotype	*CC*	56 (67.5)	59 (85.5)	1	Reference
	CT+TT	27 (32.5)	10 (14.5)	0.35 (0.16-0.79)	0.01*

Bivariate analysis of predictor variables in numerical data showed that the duration since diabetes diagnosis (in years) had a significant association with DPN [5(0-35) vs 3(0-25); p = 0.01] and body mass index had a significant association with *CC* genotype [27.23 ± 4.66 vs 25.28 ± 3.93; p = 0.023)]. Another variable such as HbA1C level, total cholesterol, LDL, HDL, triglyceride, and body mass index failed to show statistical significance. One subject is excluded in analysis involving VEGF-A level due to an extreme outlier. A trend towards a higher level of plasma VEGF-A level in DPN group when

compared with non DPN group was observed [159.50 (20.40-886.00) vs 136.10 pg/mL (17.50-631.40)] as well as between *CT+TT* vs CC genotype [166.10 (17.50-522.50) vs 133.50 (20.40-886.00)] but not statistically significant (Table 3).

Table 3: Bivariate analysis on the association of predictor variables with DPN and genotype of VEGF Gene 936 C/T polymorphism

Variable	DPN Status		p	Genotype		
	Non-DPN	DPN		*CC*	*CT+TT*	P
Diabetes	3.00	5.00	0.001	4.00	4.00	0.755
Diagnosis duration (years)	(0-25)	(0-35)		(0-35)	(0-25)	
HbA1C (%)	7.5 (5.0-15.0)	8.0 (4.0-15.0)	0.110	7.8 (4.9-15.0)	7.6 (4.0-14.9)	0.526
Total Cholesterol (mg/dL)	190 (123-287)	189 (92-346)	0.517	189 (92-346)	193 (123-249)	0.854
LDL (mg/dL)	115 ± 30	120 ± 39	0.347	119 ± 35	112 ± 33	0.305
HDL (mg/dL)	43 (30-85)	42 (30-83)	0.119	42 (30-83	44 (30-85)	0.149
Tryglyceride (mg/dL)	129 (65-636)	131 (67-330)	0.530	130 (67-636)	131 (65-265)	0.940
BMI (kg/m^2)	26.95 ± 4.73	26.53 ± 4.37	0.573	27.23 ± 4.66	25.28 ± 3.93	0.023
VEGF – A (pg/mL) (n = 151)	136.1 (17.50-631.40)	159.50 (20.40-886.00)	0.202	133.50 (20.40-886.00)	166.10 (17.50-522.50)	0.171

Multivariate logistic regression showed that plasma VEGF-A level (OR = 1.003 (1.000-1.007); p = 0.03), duration since diabetes diagnosis (OR = 1.108; 95%CI = 1.045-1.175; p = 0.001), and *CT+TT* genotype (OR = 0.347; 95%CI = 0.148-0.817; p = 0.013) were associated with DPN (Table 4).

Table 4: Multivariate logistic regression of to predict DPN in all subjects (n = 151)

Predictors	B	SE	Wald	OR (95% CI)	p
VEGF-A	0.003	0.002	4.684	1.003 (1.000-1.007)	0.03
CT+TT Genotype	-1.057	0.436	5.865	0.347 (0.148-0.817)	0.015
Diagnosis duration	0.103	0.030	11.805	1.108 (1.045-1.175)	0.001

Sub-group analysis on subjects with HbA1C level ≥ 7 (Table 5) indicated a similar relationship, showing that VEGF-A (OR = 1.011; 95%CI = (1.004-1.017; p = 0.03), diabetes duration (OR = 1.245; 95% CI = 1.117-1.388; p < 0.001), *CT+TT* genotype (OR = 0.259; 95%CI = 0.074-0.911, p = 0.035), with an adition of HDL (OR = 0.916; 95% CI = 0.857-0.978; p = 0.009) were significant predictors of DPN while LDL (OR = 1.017; 95% CI = 1.000-1.035; p = 0.053) acted as modifying factor.

Table 5: Multivariate logistic regression to predict DPN in subjects with HbA1C ≥ 7 (n = 98)

Predictors	B	SE	Wald	OR (95% CI)	p
VEGF-A	0.011	0.003	10.061	1.011 (1.004-1.017)	0.001
CT+TT Genotype	-1.349	0.614	4.433	0.259 (0.074-0.911)	0.035
Diagnosis duration	0.219	0.055	15.682	1.245 (1.117-1.388)	<0,001
HDL	-0.088	0.034	3.735	0.916 (0.857-0.978)	0.009
LDL	0.017	0.009	0.628	1.017 (1.000-1.035)	0.053

Discussion

Of all 152 subjects, in regards with polymorphism of *VEGF* gene *936 C/T*, we found that the frequency of *CC* genotype was 75.6% and *CT+TT* genotype was 24.4%. The frequency of genotype variation (*CC* vs *CT+TT*) for polymorphism of *VEGF* gene *936 C/T* (rs3025039) in general population is 75.1% vs 24.9%, East Asia 68.9% vs 31.1%, and South Asia 79.1% vs 20.1% [13]. In this study, it was observed that the genotype frequency of *VEGF* gene *936 C/T* polymorphism was close to the general population. Hardy-Weinberg analysis showed $\chi 2$ = 0.07; p = 0.787 meaning that the genotype frequency fulfilled the Hardy-Weinberg Equilibrium.

This study found that the *CT+TT* genotype of *VEGF* gene *936 C/T* polymorphism is associated with the decreased odds of DPN, hence can be considered as having a protective effect. This finding is consistent with a previous study performed by Zhang et al. (2014) [6], in which the *CC* genotype was correlated with a higher level of plasma VEGF-A. The exact role of *VEGF* gene *936 C/T* polymorphism in DPN remains controversial. Another study performed by Kim et al. (2009) [7] found that there was no significant relationship between *VEGF* gene *936 C/T* polymorphism and DPN although DPN and *TT* genotype correlated with higher plasma VEGF-A level. Another Single Nucleotide Polymorphism (SNP) regarding *VEGF* gene at the position of *7C/T* found to be associated with DPN. The allele *C* was considered probably related to increased risk of developing DPN while allele *T* might be protective [14]. The exact mechanism of the potentially protective effect of *T* allele in this SNPs is still unclear [6] [14].

Although this study failed to reach a statistical significance, a higher median of VEGF-A level was observed in subjects with DPN and *CT+TT* genotype. These findings support the results of the study by Kim et al., (2009) [7]. It is presumed that the *VEGF* gene 936 C/T polymorphism is somehow related with the AP-4 binding protein of VEGF gene which determines the level of circulating VEGF. Deguchi et al., (2009) revealed that the circulating VEGF level increased in the early and symptomatic stage of DPN and decreased later at an advanced stage [5]. The positive relationship between VEGF-A level and DPN from multivariate logistic regression analysis was found in this study supports this finding.

Another interesting finding from this study is the significant association between polymorphism of *VEGF* gene *936 C/T* with body mass index. *CC* genotype had a significantly higher BMI compared with *CT + TT* genotype (27.23 ± 4.66 vs 25.28 ± 3.93; p = 0.023) (Table 2). The previous study on obstructive sleep apnea failed to prove the relationship between *VEGF* gene *936 C/T* polymorphism with obesity [15]. High VEGF-A level and VEGF-A overexpression are potentially protective towards obesity and insulin resistance. VEGF-A also has a role on the thermogenesis of brown adipose tissue (BAT) which plays a role in the development of obesity [16]. This study found that the level of VEGF-A on *CT + TT* genotype is higher compared with CC genotype. Although the difference was not statistically

significant, this could somehow explain the relationship between *VEGF* gene *936 C/T* polymorphism with BMI. More studies are still needed to confirm this finding

The increase in VEGF expression in the early stage of DPN is a response to hypoxia and oxidative stress. The target of this process is the endothelial cells, with proliferation and angiogenesis/ neovascularisation as a result. VEGF through VEGFR2 also plays the neuroprotective role together with other angioneurins such as IGF1, TGF beta 1, EPO, FGF2, HGF, EGF, and PGRN. These substances are believed to have a positive effect on remyelination and neurogenesis. These facts support the concept that VEGF has a dual effect, involved in pathogenesis as well as protecting the nerves in DPN [5] [17]. The fact that VEGF-A had a positive but small effect on the relationship with the occurrence of DPN could be explained by this concept.

The regression model in this study showed that the interaction of *VEGF* gene *936 C/T* polymorphism with diagnosis duration and vascular factors especially blood lipids plays an important role in the development of DPN. In subjects with HbA1C ≥ 7%, LDL, HDL, and duration of diabetes diagnosis had a positive effect on the development of PDN. These findings have a clinical/practical implication, meaning that prevention strategy can be focused on individuals with high HbA1C, high LDL level, low HDL level, and longer duration of diabetes diagnosis.

Dislipidemia considered as an important risk factor of DPN [18]. Lipid levels are related with the accumulation of sorbitol, formation of oxidised lipids and PARP, and activation of lipoxygenase on peripheral nerve fibres. Free fatty acids cause toxicity on neuronal cell and Schwann's cells and also trigger the release of proinflammatory cytokines causing neuronal inflammation. Lipids are also related to insulin resistance of which in type-2 diabetic patients may worsen the hyperglycemic state. The peripheral nerve tissue expressed oxidised LDL (oxLDL) scavenger receptor. The receptors internalise oxLDL and glycated LDL which triggers inflammatory signals, causing NADPH oxidase inactivation which may cause increased oxidative stress [19] [20]. Lower HDL level has been proven to be associated with DPN and means that management to dislipidemia plays an important role in preventing and treatment of DPN [21].

To our knowledge, this is the first study in regards to *VEGF* gene *936 C/T* polymorphism in a diabetic subject in Indonesia. There were some limitations to this study. Outliers of the VEGF-A level might show that excluding conditions, of which some of them relied only on medical record, such as acute on chronic OA, history of cancer, acute stroke and acute myocardial infarction were not enough to avoid extreme values of VEGF-A level. It is necessary to add the items on exclusion criteria such as smoking (active or passive), alcoholism, and other situation that could interfere with VEGF-A level. The diagnostic criteria in this study were likely only to accommodate mild DPN. Hence the result of this study can only be applied to those group. This study did not compare the genotype frequency between diabetic and normal subjects. Nevertheless, some previous studies have proved that polymorphism of *VEGF* gene *936 C/T* did not associate with diabetes mellitus [6] [22].

The *CT+TT* genotype of *VEGF* gene *936 C/T* polymorphism is potentially protective, while the *CC* genotype may be associated with a higher risk of developing DPN. A trend of higher VEGF-A level in DPN and *CT + TT* genotype was observed. This study also suggested that VEGF-A has a potential role in the pathogenesis of DPN, especially from the vascular aspect. Vascular risk factors, especially HbA1C and LDL, and HDL, as well as diagnosis duration, acted as modifying factors which interact with *VEGF* gene *936 C/T* polymorphism in the development of DPN.

Further study is needed to reveal the relationship between *VEGF* gene *936 C/T* polymorphism and the physiological function of peripheral nerve in DPN such as quantitative sensory testing or neuroesthesiometer value of each nerve. This is important to find out the influence of *VEGF* gene *936 C/T* polymorphism on the physiology of peripheral nerve to reveal its protective mechanism. Further study towards other genetic variants related to glycemic and metabolic function, ethnicity, especially the ones presumably related to nerve structure and function is needed to find the strongest genetic factors associated with DPN.

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