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RESEARCH ARTICLE

Single Nuclear Polymorphisms of VEGF, TGF-β1, MMP9 Genes in Type 2 Diabetic Foot Ulcer Patients in Indonesian Population: A Case Control Study

Dedy Pratama[1], Akhmadu Muradi[1], Dwi A Suryandari[2], Patrianef[1], Setyawati B[3], Kemas M Dahlan[4], Igab Krisna Wibawa[5], Tom Ch Adriani[6]

1 Department of Surgery, Faculty of Medicine Universitas Indonesia – Cipto Mangunkusumo Hospital.

2 Department of Medical Biology, Faculty of Medicine Universitas Indonesia.

3 Department of Community Medicine, Faculty of Medicine Universitas Indonesia.

4 Department of Surgery, Faculty of Medicine, Universitas Sriwijaya – Mohammad Hoesin Hospital, Palembang.

5 Department of Surgery, Faculty of Medicine, Universitas Udayana - Sanglah Hospital, Denpasa.r

6 Department of Surgery, Faculty of Medicine, Universitas Hasanuddin - Tadjuddin Chalid Hospital, Makassar.

***Corresponding Author:** *Dedy Pratama*

Abstract

Objective: Vascular endothelial growth factor (VEGF), transforming growth factor beta 1 (TGF-β1) and matrix metalloprotein-9 (MMP9) are known to have roles in the process of diabetic foot ulcer (DFU) formation. VEGF rs2010963C>G rs1271283232T>C, TGF-β1 rs1982073C>T rs1800469C>T, and MMP9 rs3918242C>T rs367601348A>G genes polymorphism may result in differences in the quantity and quality of the proteins which influence the risk of DFU formation. This study aims to assess the difference in frequency distribution of certain VEGF, TGF-β1, and MMP9 genes polymorphism between diabetic patients with and without DFU. Methods: A case-control study was conducted among patients with type-2 DM with DFU (case) and without DFU (control) in Cipto Mangunkusumo Hospital Jakarta, with DNA analysis using Polymerase Chain Reaction Restriction Fragment Length Polymorphism (PCR-RFLP) technique. The confounding factors are also analyzed. Results: A total of 197 patients was assessed, 96 with DFU and 101 control. The genotype analysis by logistic regression found significant association of CT genotype in TGF-β1 rs1982073C>T (OR:0.28;95%CI:0.13-0.60;p=0.001 compared with CC); TT genotype in TGF-β1 rs1800469C>T, (OR:2.37;95%CI:1.11-0.60;p=0.001 compared with CC); TC genotype in MMP9 rs3918242C>T (OR:0.19;95%CI:0.19-0.64;p=0.001 compared with CC). There are no significant association in any mutation in VEGF rs2010963C>G, rs1271283232T>C, and MMP9 rs367601348A>G. Conclusion: VEGF rs2010963C>G rs1271283232T>C, and MMP9 rs367601348A>G polymorphisms did not have significant association with DFU formation. CT in TGF-β1 rs1982073C>T and TC in MMP9 rs3918242C>T found as protective factor for DFU, and TT in TGF-β1 rs1800469C>T as risk factor for DFU.

Keywords: *Diabetic foot ulcer, Genetic polymorphism, VEGF, MMP9, TGF-β1, rs2010963C>G, rs1271283232T>C, rs1982073C>T, rs1800469C>T, rs3918242C>T rs367601348A>G*

Introduction

One of the severe complications that may potentially affect patients with diabetes mellitus (DM) is a diabetic foot ulcer (DFU). Terminologically, DFU represents all foot lesions occurring as an aftermath of diabetes or its complications [1]. DFU is one of the main causes of hospitalization in DM patients [1, 2]. In developing countries, the incidence of DM among the elderly is very high. Every year, 4 million people with DM develop diabetic foot complications. Around the world, one case requiring amputation due to DM occurs every 30 seconds [1]. DFU develops as a result of chronic

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hyperglycemia in DM that leads to neuropathy, trauma, infection, and ischemia (ankle-brachial index [ABI] score less than 0.7, toe pressure <40 mmHg, transcutaneous oxygen tension [$TcPO_2$] <30 mmHg) [3-5].Neuropathy, whether it be sensoric, motoric or autonomic will induce various changes in the skin, muscles, tendons, and bones, creating a change of pressure distribution over the soles of the feet, facilitating the formation of ulcers [3-5].

Neuropathy, recurrent trauma, poor limb vascularization and infection will affect DFU formation and influence wound healing in patients with DM [6].Vascular endothelial growth factor (VEGF) have significant role in mediating micro vascular complication in DM such as diabetic retinopathy, and neuropathy.

VEGF mitogenic effect on endothelial and non-endothelial cell are thought to be the mechanism in which VEGF contribute to DM complications [7-11]. VEGF plays an angiogenic role in the formation of microcirculation, as demonstrated in Peripheral Arterial Disease (PAD) and Critical Limb Ischemia (CLI) [7].Higher level of VEGF has been associated with type 2 DM and its complication, and polymorphism is one of the factors that regulate VEGF levels and production [11, 12].

VEGF rs2010963C>G is a polymorphism of 5' prime untranslated (UTR) region. The polymorphism at +405 predicted to lie within a potential myeloid zinc finger protein (MZF1) binding site in which C allele reduce the binding specificity of this transcription factor binding motifs. Thus, G allele associated with increases VEGF production [12].

VEGF rs1271283232 T>C is a polymorphism located in regulatory region of VEGF gene. C allele of VEGF rs1271283232 gene associated with higher level of VEGF, while T allele reduces the production of VEGF [11, 12].Transforming growth factor-B1 (TGF-β1) is a pleiotropic cytokine that plays a key immunoregulatory role in the activation of inflammation and resolution of the inflammatory response as a variation of autoimmune diseases. Increased level of glucose induces the rise of TGF-β1 level.

TGF-β1 regulates the production of almost all extracellular matrix (ECM) molecules. From the said evidence, the expression of TGF-β1 is associated with the risk of developing DM and its complications [12-14].Number of studies have been conducted to evaluate the relationship between TGF-β1 polymorphism and the risk of developing DM and its complication. However, the results are still within reach of the conflicts that have previously been reported by prior studies [15-17].

Some of the known polymorphism in TGF-β1 genes are TGF-β1 rs1982073 and TGF-β1 rs1800469. TGF-β1 rs1982073C>T is a single nucleotide polymorphism (SNP) located in the first exon of the TGF-β1 gene. The C allele of TGF-β1 rs1982073 gene that codes proline has been associated with microvascular complication of DM, such as nephropathy, retinopathy and also neuropathy that is the main cause of DFU formation [16].

TGF-β1 rs1800469, also known as -509 C>T, is an SNP in the promoter region of TGF-β1 gene. This SNP does not change the nature of the TGF-β1 protein, instead it changes the amount of the protein produced.

The T allele increases the amount of TGF-β1 produced, by preventing AP1 from binding to this region where the C allele would normally down regulate production [17].Matrix Metalloprotein 9 (MMP9) is a form of gelatinase that degrades ECM protein, playing an important role in vascular remodeling. Genetic abnormalities can disrupt MMP9 protein synthesis, indirectly affecting the process of wound healing. MMP9 gene polymorphism is strongly thought to influence the process of ulcer formation and wound healing in patients with DFU [16, 17].The most common variation of MMP9 is MMP9 rs3918242C>T SNP also known as -1562C>T, even though the output is still controversial.

Several studies indicate that the function of MMP9 rs3918242C>T polymorphism promoter is associated with DFU. However, this has not been confirmed by other studies, calling interest for further investigations.[14] Other known SNP is MMP9 rs367601348 that located in coding sequence of MMP9 gene. MMP9

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rs367601348 is a missense variant in which A allele codes Threonine (AAG), while G allele codes Alanine (GAG) although the phenotype effects of this polymorphism have not been observed yet.[19] Hence, we want to study the genes distribution of VEGF rs2010963C>G, rs1271283232T>C, TGF B-1 rs1982073C>T, rs1800469C>T, and MMP9 rs3918242C>T, rs367601348A>G and its association with DFU.

Methods

A case-control study was conducted with the aim to assess the frequency distribution of vascular endothelial growth factor (VEGF) rs2010963C>G rs1271283232T>C, transforming growth factor beta 1 (TGF-β1) rs1982073C>T rs1800469C>T and matrix metalloprotein 9 (MMP9) rs3918242C>T rs367601348A>G genes polymorphism and their association with diabetic foot ulcer (DFU) in type-2 diabetic patients in Cipto Mangunkusumo Hospital. This study was conducted under the Division of Vascular and Endovascular Surgery Faculty of Medicine Universitas Indonesia (FMUI)-Cipto Mangunkusumo Hospital Jakarta in collaboration with Biomolecular Microbiology Laboratory FMUI-Cipto Mangunkusumo Hospital Jakarta.

Blood samples were obtained from patients selected with consecutive sampling technique within time limit between September-December 2016 with minimum samples of 85 in each group. All type-2 DM patients with DFU managed in Cipto Mangunkusumo Hospital who met the inclusion and exclusion criteria were recorded and categorized into a case group (patients with DFU) and control group consists of type-2 DM patients without DFU. DNA extraction from peripheral blood samples conducted using Wizard® Genomic DNA Purification Kit according to Isolating Genomic DNA from Whole Blood (3 ml Sample Volume) procedure.

The VEGF rs2010963C>G gene PCR was performed as follows: The chosen F primer was 5' GGG CGG TGT CTG TCT GTC TG 3' and R primer was 5' CGA CGG CTT GGG GAG ATT GC 3'. PCR temperature was set at 95°C,for 3 minutes; 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds; and 72°C for 7 minutes results in 237 bp band. RFLP procedure using BsmF1 enzyme at 65°C for 60 minutes.

The result of PCR-RFLP was expected to show a CC genotype band at distances of 170 bp and 103 bp, GG genotype at 273 bp, and CG genotype at 273 bp, 170 bp, and 103 bp. PCR of VEGF rs271283232 T>C gene was performed as follows: using F primer of 5' CCT CTT TAG CCA GAG CCG GGG 3', and R primer of 5' TGG CCT TCT CCC CGC TCC GAC 3'. PCR temperature was set at 95°C,for 3 minutes; 95°C for 30 seconds, 66°C for 30 seconds, 72°C for 30 seconds; and 72°C for 7 minutes results in 176bp bands.

RFLP process used Bsa H1 as restriction enzyme the results were expected to show a CC genotype band (160bp and 16 bp), TT (176 bp) and TC (176bp, 60bp and16 bp).PCR of TGF-β1 rs1800469 C>T gene was performed as follows: using F primer of 5' GTC GCA GGG TGT TGA GTG ACA 3', and R primer of 5' AGG GGG CAA CAG GAC ACC TTA 3'. PCR temperature was set at 95°C,for 3 minutes; 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds; and 72°C for 7 minutes results in 123bp bands. RFLP process used AF1II as restriction enzyme set at 65°C for 20 minutes. The results were expected to show a CC genotype band (123bp), TT (101bp and 22bp) and CT (123bp,101bp and 22bp).

PCR of TGF-β1 rs1982073 C>T gene was performed as follows: using F primer of 5' CTC CGG GCT GCG GCT GCA GC 3', and R primer of 5' GGC CTC GAT GCG CTT CCG CTT CA 3'. PCR temperature was set at 95°C,for 3minutes; 95°C for 30 seconds, 66°C for 30 seconds, 72°C for 30 seconds; and 72°C for 7 minutes results in 139bp bands. RFLP process used PvuII. The results were expected to show a CC genotype band (139bp), TT (117bp and 22bp) and CT (139bp, 117bp and 22bp). PCR of MMP9 rs3918242C>T gene was performed as follows: using F primer of 5' GCC TGG CAC ATA GTA GGC CC 3', and R primer of 5' CTT CCT AGC CAG CCG GCA TC 3'.

PCR temperature was set to perform 30 cycles in 1 minute at 94°C, 1 minute at 58°C, and 1 minute at 72°C results in 436 bp bands. RFLP process used Sph1 as restriction enzyme set at 58°C for 60 minutes. The results were expected to show a CC genotype band (436bp), TT (224bp and 192bp) and CT (436bp, 224bp and 192bp).

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Polymerase chain reaction (PCR) amplification of MMP-9 rs367601348A>G was done at mutation region using an existing primer with forward primer (5′-GCC TGG CAC ATA GTA GGC CC-3′) and reverse primer (5′-CTT CCT AGC CAG CCG GCA TC-3′) at the promoter sequence of MMP-9 gene. PCR was set to perform 30 cycles in 1 minute at 94°C, 1 minute at 58°C, and 1 minute at 72°C and produced 439bp. The prepared 439-bp fragment was dissolved with Sph1 enzyme at 37°C for 1 hour. When a A>G transition occurred, the formed 439-bp fragment split into 2 sub-fragments, 252 bp and 187 bp.

Results

The total study sample consisted of 197 patients, 96 patients (49%) representing DM patients with DFU and 101(51%) representing DM patients without DFU. Characteristics patients for each group showed in the Table 1. In total, sexes distribution are 49.2% male and 50.8% female, with an age average of 56.33 years, ranging from 34 to 90 years. Using Chi Square analysis, significant characteristic difference between group found in peripheral neuropathy (DFU+:93.8%, DFU-: 61.4%, p<0.001), rest pain (DFU+:34.4%, DFU-:4%, p<0.001), Smoking (DFU+:57.3%, DFU-: 30.7%, p<0.001), hypertension (DFU+:60.4%, DFU-: 36.6%, p<0.001), and peripheral artery disease (DFU+:56.2%, DFU-: 10.9%, p<0.001). Laboratory analysis of the blood samples also show significant difference between group for anemia determined (DFU+:37.5%, DFU-: 12.9%, p<0.001), Leukocytosis (DFU+:77.1%, DFU-: 31.7%, p<0.001), and Hyperglycemia (DFU+:56.2%, DFU-: 40.6%, p<0.001).

Risk of DFU increased by these factors are as follows: Diabetic neuropathy (OR; 9.43, 95% CI; 3.76-23.63 and p<0.001); hypertension (OR; 2.64, 95%CI; 1.48-4.69 and p=0.001); smoking (OR 3.02, 95%CI; 1.68-5.43 and p<0.001); PAD (OR;10.51, 95%CI; 4.99-22.15 and p<0.001); anemia (OR;4.06, 95%CI; 1.98-8.29 and p<0.001); leukocytosis (OR;7.25, 95% CI; 3.84-13.67 and p<0.001); and hyperglycemia (OR;1.88, 95% CI; 1.06-3.31 and p<0.001).

Table. 1: Demographic characteristics of patients with diabetic foot ulcer (DFU) Type-2 DM in Cipto Mangunkusumo Hospital during the Period of September to December 2016 (n=197)

Variable	DFU (+) n=96		DFU (-) n=101		OR	95% CI	p*
	n	%	n	%			
Age (years)							
≥ 70	8	8.3	6	5.9	1.26	0.37 – 4.33	0.707lr
60 – 69	29	30.2	23	22.8	1.18	0.52 – 2.75	0.671lr
50 – 59	39	40.6	53	52.5	0.69	0.33 – 1.48	0.351lr
< 50	20	20.8	19	18.8	Ref		
Sex (male)	48	50.0	49	48.5	1.06	0.60 – 1.85	0.835cs
Ethnicity							
Jawa	70	72.9	74	73.3	1.32	0.34 – 5.12	0.687lr
Sumatera	21	21.9	23	22.8	0.96	0.49 – 1.89	0.918lr
Others	5	5.2	4	4.0	Ref		
Duration of DM							
≥ 10	52	54.2	46	45.5	1.52	0.75 – 3.07	0.237lr
5 – 9	24	25.0	28	27.7	1.15	0.52 – 2.56	0.719lr
< 5	20	20.8	27	26.7	Ref		
BMI							
Overweight	18	18.8	21	20.8	0.98	0.41 – 2.35	0.974lr
Normoweight	58	60.4	57	56.4	1.17	0.58 – 2.36	0.661lr
Underweight	20	20.8	23	22.8	Ref		
Neuropathy	90	93.8	62	61.4	9.43	3.76 -23.63	<0.001cs
Rest pain	33	34.4	4	4.0	12.70	4.29 – 37.59	<0.001cs
Smoking	55	57.3	31	30.7	3.02	1.68 – 5.43	<0.001cs
Hypertension	58	60.4	37	36.6	2.64	1.48 – 4.69	0.001cs
PAD	54	56.2	11	10.9	10.51	4.99 – 22.15	<0.001
Anemia	36	37.5	13	12.9	4.06	1.98 – 8.29	<0.001
Leukocytosis	74	77.1	32	31.7	7.25	3.84 – 13.67	<0.001
Hyperglycemia	54	56.2	41	40.6	1.88	1.06 – 3.31	0.028

*Chi Square analysis

The total frequency distribution of VEGF rs2010963C>G is 7.6% wild-type CC, 71.6%

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mutant heterozygote CG, and 20.8% mutant homozygote GG. In total, the mutant genotype compose 92.4% of genotype. Allele analysis found 223(56.6%) mutant G allele, and 171 (43.4%) C allele. The genotypic distribution of the VEGF rs1271283232T>C gene was 14.2% mutant CC, 39.1% mutant heterozygote TC, and 46% wild type homozygote TT. Allele distributions shows T as wild type allele composed 261(66.2%) of total allele, and 133(33.8) of C allele.

The total frequency distribution of TGF-ß1 rs1982073C>T is 60.4% wild-type CC, 23.9% mutant heterozygote CT, and 15.7% mutant homozygote TT. Allele analysis found 109(27.7%) mutant T allele, and 285 (72.3%) C allele. The genotypic distribution of the TGF-ß1 rs1800469C>T gene was 33.5% wild-type CC, 41.6% mutant heterozygote CT, and 24.9% mutant homozygote TT. Allele distributions shows T as a mutant allele composed 180(45.7%) of total allele, and 214(54.3) of C allele. Frequency distribution of MMP9 rs3918242C>T is 51.8% wild-type CC, 45.7% mutant heterozygote CT, and 2.5% mutant homozygote TT. In total, the mutant genotype compose 48.2% of genotype. Allele analysis found 294 (74.6%) mutant T allele, and 100(25.4%) C allele.

The genotypic distribution of MMP9 rs367601348A>G gene was 17.3% wild-type AA, 48.2% mutant heterozygote AG, and 34.5% mutant homozygote GG. Allele distributions shows G as a mutant allele compose 231(58.6%) of total allele, and 163(41.4%) of C allele. The frequency distributions of the genes for DFU+ and DFU- groups are presented in (Table 2).

Table 2: Distribution of Genotypes and Alleles in VEGF rs2010963C>G, VEGF rs1271283232T>C, TGF B-1 rs1982073C>T, TGF B-1 rs1800469C>T, and MMP9 rs3918242C>T, MMP9 rs367601348A>G Gene Polymorphisms in Patients with Diabetic Foot Ulcer in Cipto Mangunkusumo Hospital

Gene	Polymorphism		DFU (+) n=96		DFU (-) n=101		Total n=197	
			n	%	n	%	N	%
VEGF	rs2010963C>G	GG	18	18.8	23	22.8	41	20.8
		CG	69	71.9	72	71.3	141	71.6
		CC	9	9.4	6	5.9	15	7.6
		G	105	54.7	118	58.4	223	56.6
		C	87	45.3	84	41.6	171	43.4
	rs1271283232T>C	CC	13	13.5	15	14.9	28	14.2
		TC	41	42.7	36	35.6	77	39.1
		TT	42	43.8	50	49.5	92	46.7
		C	67	34.9	66	32.7	133	33.8
		T	125	65.1	136	67.3	261	66.2
TGF-ß1	rs1982073C>T	CC	65	67.7	54	53.5	119	60.4
		CT	12	12.5	35	34.7	47	23.9
		TT	19	19.8	12	11.9	31	15.7
		C	142	74.0	143	70.8	285	72.3
		T	50	26.0	59	29.2	109	27.7
	rs1800469C>T	CC	25	26.0	41	40.6	66	33.5
		CT	42	43.8	40	39.6	82	41.6
		TT	29	30.2	20	19.8	49	24.9
		C	92	47.9	122	60.4	214	54.3
		T	100	52.1	80	39.6	180	45.7
MMP9	rs3918242C>T	CC	63	65.6	39	38.6	102	51.8
		TC	33	34.4	57	56.4	90	45.7
		TT	0	0	5	5.0	5	2.5
		C	159	100	135	66.8	294	74.6
		T	33	0	67	33.2	100	25.4

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	rs367601348A>G	AA	19	19.8	15	14.9	34	17.3
		AG	39	40.6	56	55.4	95	48.2
		GG	38	39.6	30	29.7	68	34.5
		A	77	40.1	86	42.6	163	41.4
		G	115	59.9	116	57.4	231	58.6

We conduct bivariate analysis of the genotype and allele of VEGF rs2010963C>G, VEGF rs1271283232T>C, TGF B-1 rs1982073C>T, TGF B-1 rs1800469C>T, and MMP9 rs3918242C>T, MMP9 rs367601348A>G gene polymorphisms to see the association of each polymorphism and DFU occurrence in patients. We compared mutant genotype with the wild type genotype as reference.

There is no statistically significant difference of DFU risk between each mutant genotype of VEGF rs2010963C>G and VEGF rs1271283232T>C compared to the wild type. We found statistically significant difference of CT genotype of TGF-β1 rs1982073C>T compared to the wild type CC where CT genotype is a protective factor of DFU (OR: 0.28, 95%CI: 0.13-0.60, p=0.001). TT genotype of TGF-β1 rs1800469C>T is a risk factor of DFU compared to CC genotype (OR: 2.3, 95%CI: 1.11-5.06, p=0.025). MMP9 genes bivariate analysis found CT genotype of MMP9 rs3918242C>T is a protective factor of DFU compared to CC genotype (OR: 0.35, 95%CI: 0.19-0.64, p=0.001), while there is no significant difference found in MMP9 rs367601348A>G genes polymorphism. (Table 3).Bivariate analysis also conducted for the alleles.

There is no statistically significant difference found for VEGF rs2010963C>G and rs1271283232T>C alleles. T allele in TGF-β1 rs1800469 is a risk factor of DFU compared to C allele (OR: 1.65, 95%CI: 1.11-2.47, p=0.017). CT genotype of TGF-β1 rs1982073 is statistically significant, however, no statistically significant difference found when comparing T allele with C allele (OR: 0.85, 95%CI: 0.55-1.33, p=0.554). T allele in MMP9 rs3918242 is a protective factor of DFU compared to C allele (OR: 0.41, 95%CI: 0.26-0.67, p=0.001). There is no statistically significant difference of allele comparison found for MMP9 rs367601348A>G (table 3).

Table 3: Bivariate Analysis of Genotypes and Alleles in VEGF rs2010963C>G, rs1271283232T>C, TGF B-1 rs1982073C>T, rs1800469C>T, and MMP9 rs3918242C>T, rs367601348A>G Gene Polymorphisms in al (n=197)

Genes	polymorphisms		DFU (+) n=96		DFU (-) n=101		OR	95% CI	p*
			n	%	n	%			
VEGF	rs2010963	GG	18	18.8	23	22.8	0.52	0.15 – 1.73	0.289
		CG	69	71.9	72	71.3	0.63	0.21 – 1.89	0.418
		CC	9	9.4	6	5.9	Ref		
		G	105	0.55	118	0.58	0.86	0.57-1.28	0.456
		C	87	0.45	84	0.42	Ref		
VEGF	rs1271283232	CC	13	13.5	15	14.9	1.03	0.44 – 2.41	0.88
		CT	41	42.7	36	35.6	1.36	0.74 – 2.49	0.406
		TT	42	43.8	50	49.5	Ref		
		C	67	0.35	66	0.33	1.10	0.72-1.68	0.718
		T	125	0.65	136	0.67	Ref		
TGFβ 1	rs1982073	TT	19	19.8	12	11.9	1.3	0.58 – 2.95	0.506
		CT	12	12.5	35	34.7	0.28	0.13 – 0.60	0.001
		CC	65	67.7	54	53.5	Ref		
		T	50	26.0	59	29.2	0.85	0.55-1.33	0.554
		C	142	74.0	143	70.8	Ref		
TGFβ 1	rs1800469	TT	29	30.2	20	19.8	2.37	1.11 – 5.06	0.025

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		CT CC	42 25	43.8 26.0	40 41	39.6 40.6	1.72 Ref	0.89 – 3.33	0.106
		T	100	52.1	80	39.6	1.65	1.11-2.47	0.017
		C	92	47.9	122	60.4	Ref		
MMP9	rs3918242C>T	TT TC CC	0 33 63	0.0 34.4 65.6	5 57 39	5.0 56.4 38.6	– 0.35 Ref	– 0.19 – 0.64	0.999 0.001
		T	33	17.2	67	33.2	0.41	0.26-0.67	0.001
		C	159	82.8	135	66.8	Ref		
MMP9	rs367601348	GG AG AA	38 39 19	39.6 40.6 19.8	30 56 15	29.7 55.4 14.9	1.00 0.55 Ref	0.43 – 2.29 0.24 – 1.21	1.000 0.138
		G	115	59.9	116	57.4	1.11	0.86-1.30	0.689
		A	77	40.1	86	42.6	Ref		

*Chi square

To analyze the interaction of the specified genotype polymorphism of VEGV, TGF-ß1 and MMP-9 genes, we conducted multivariate analysis using binary logistic regression. After taking into consideration the interaction of all polymorphism studied, we found statistically significant results, in which CT genotype in TGF-ß1 rs1982073C>T is a protective factor of DFU compared to CC genotype. (OR: 0.29; 95%CI: 0.12-0.69, p=0.005). We also found that TT genotype in rs1800469C>T is a risk factor of DFU (OR: 3.18; 95%CI: 1.27-7.96, p=0.013). CT genotype in MMP9 rs3918242C>T polymorphism is a protective factor of DFU in diabetic patients compared to those with CC genotype (OR: 0.36, 95%CI: 0.19-0.69, p: 0.002). We found no statistically significant results for the rest of gene polymorphism studied (Table 4).

Table 4: Multivariate Analysis of Genotypes in VEGF rs2010963C>G, VEGF rs1271283232T>C, TGF B-1 rs1982073C>T, TGF B-1 rs1800469C>T, and MMP9 rs3918242C>T, MMP9 rs367601348A>G Gene Polymorphisms in Patients with Diabetic Foot Ulcer in Cipto Mangunkusumo Hospital (n=197)

Gene	Polymorphism		DFU (+) n=96		DFU (-) n=101		OR (Exp(B))	95% CI	p*
			n	%	n	%			
VEGF	rs2010963	GG CG CC	18 69 9	18.8 71.9 9.4	23 72 6	22.8 71.3 5.9	0.53 0.91 Ref	0.13 – 2.07 0.26 – 3.12	0.366 0.885
VEGF	rs1271283232	TT CT CC	42 41 13	43.8 42.7 13.5	50 36 15	49.5 35.6 14.9	1.24 1.24 Ref	0.47 – 3.26 0.46 – 3.29	0.652 0.667
TGF	rs1982073	TT CT CC	19 12 65	19.8 12.5 67.7	12 35 54	11.9 34.7 53.5	1.81 0.29 Ref	0.72 – 4.57 0.12 – 0.69	0.206 0.005
TGF	rs1800469	TT CT CC	29 42 25	30.2 43.8 26.0	20 40 41	19.8 39.6 40.6	3.18 1.79 Ref	1.27 – 7.96 0.82 – 3.87	0.013 0.138
MMP	rs3918242	TT CT CC	0 33 63	0.0 34.4 65.6	5 57 39	5.0 56.4 38.6	– 0.36 Ref	– 0.19 – 0.69	0.999 0.002
MMP	rs367601348	GG AG AA	38 39 19	39.6 40.6 19.8	30 56 15	29.7 55.4 14.9	0.91 0.50	0.35 – 2.31 0.20 – 1.21	0.842 0.127

*Binary logistic regression

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Discussion

From the subject characteristics of patients in this study, the relationship between type-2 DM and sex is not significantly (p=0.835; p>0.05) different from the study by K Singh et al. (2013) that involved more men (85%) and fewer women (15%) [21]. Type-2 DM patients with DFU aged 50-59 years dominated other age groups, in line with a report from Hicks et al (2016) that stated an increased risk of DFU as age advances [22]. In this study, BMI was statistically classified as underweight, normoweight, and overweight.

Overweight and obesity are known to be associated with thewith type-2 DM, and obesity is known to increases the risk of microvascular complications, associated with the rise of HbA1C, LDL, and systolic blood pressure. We find no statistically significant difference of the DFU occurrence in BMI groups in this study. On the other hand, smoking and DM comorbidities such as hypertension are also found to increase the chance of DFU formation in type 2 DM patients [23].Neuropathies as a results of microangiopathy in DM patients, lead to patients ignoring the presence of wounds or trauma due to disorders in pain perception. Wounds usually expand in a short period accompanied by accidental trauma [4, 7].

This is often associated with ulcer formation, which in this study was found with OR =9.43 (95%CI: 3.76-23.63) and p<0.001. Type-2 DM also results in macroangiopathy complications leading to the development of PAD (OR=10.51, p<0.05), the symptoms of which include rest pain (OR=12.70, p<0.05) and claudication. (OR=9.12, p<0.05). The statistically significant association of neuropathy and PAD strongly confirmed the important role of neuropathy and PAD in the process of DFU formation.

From hematologic characteristics, the prevalence of anemia in patients with DFU is currently increasing. This study found the average level of blood hemoglobin was 11.43 g/dl. The presence of anemia in 37.5% of patients with DFU, and only in 12.9% of patients without DFU (OR: 4, 06, 95%CI: 1.98-8.29, p<0.001). Chuan (2014) recorded the much higher incidence of anemia in DFU patients as high as 59.3%-61.8% [24]. Causes of anemia include chronic inflammation, diabetic nephropathy, and malnutrition. Chronic inflammation is said to be the most common cause of anemia in patients with diabetes [24]. DM patients managed in Cipto Mangunkusumo Hospital showed 53.8% of DM patients had WBC levels over 10,000 cells/µL with 77.1% of DM patients with DFU had a WBC level of >10,000 cells/µL.

This reason of the WBC increase (leukocytosis) thought because most patients who were managed in Cipto Mangunkusumo Hospital had suffered from diabetic ulcers for a long time and therefore had been complicated with infections. It could also be due to chronic hyperglycemic conditions, immune response disorders, neuropathy, and PAD, which could play as predisposing factors for acquiring infections.

Although random blood glucose level is not the ideal parameter to assess glycemic control in DM patients, patients with hyperglycemia during random blood glucose level measurement (blood glucose >200mg/dL) have higher probability of DFU formation compared to non-hyperglycemic patients. This support the importance of controlling blood glucose level to prevent DFU formation. Higher level of VEGF has been associated with type 2 DM and its complication [9-12].

The VEGF gene is located on chromosome 6p21.3 and consists of 8 exons exhibiting alternate splicing to form a family of proteins. VEGF rs2010963C>G is a polymorphism of 5' prime untranslated (UTR) region. The polymorphism at VEGF rs2010963was predicted to lie within a potential myeloid zinc finger protein (MZF1) binding site in which C allele reduce the binding specificity of this transcription factor binding motifs.[11,12] Thus, G allele associated with increases VEGF production.

The study by Watson (2000) also shows dose dependent VEGF production where the highest VEGF protein production was recorded for the GG genotype, intermediate for GC, and the lowest for the CC genotype [12].VEGF rs1271283232 T>C is a polymorphism located in regulatory region of VEGF gene. C allele of VEGF rs1271283232 gene associated with higher level of VEGF, while T allele reduce the production of VEGF [11].Genotypic

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distribution of VEGF rs2010963C>G gene polymorphism was found to be as follows. Wild-type CC was found to comprise 7,6%, mutant heterozygote CG 71,6% and mutant homozygote GG 20,8%. Cumulatively, 92,4% of the genotypes were found to be mutant genotypes. There was an increase of G alleles as a mutantt allele as big as 223(56.6%).

In their study identifying the correlation between rs2010963 polymorphism and markers of carotid atherosclerosis in patients with type 2 diabetes mellitus, Merlo *et al*. Reported the genotype distributions for rs2010963 polymorphisms among Caucasians as follows: CC genotype 8.7%, CG genotype 47.1%, and GG genotype 44.2% (χ^2 = 3.48; p =0.06) for those with type 2 diabetes and CC genotype 9%, CG genotype 48%, and GG genotype 43% (χ^2 =1.46; p =0.22) for subjects in their control group [25].Meanwhile, the genotypic distribution of VEGF rs1271283232T>C gene polymorphism was found to consist of 46.7% wild-type TT, 39,1% (77) mutant heterozygote CT and 14,2% (28) mutant homozygote CC. The distribution of allele T as a mutant allele in VEGF -460 T>C gene polymorphism was 261(66,2%).

Both bivariate and multivariate analysis yield statistically insignificant results. Allele analysis also failed to show statistically significant difference for both polymorphisms. TGF-β1 regulates many kinds of cell activities such as cell growth, differentiation, matrix production and fibrosis in various tissues such as heart and blood vessels. TGF-β1 is one kind of several proteins that triggers the extracellular matrix protein overproduction [13-15]. The fibrosis caused by TGF-β1 overproduction in blood vessels have a role in the microvascular and macrovascular complication of DM [14].An increased TGF-β1 production has been reported in microvascular DM complication such as diabetic nephropathy [25].

Log-transformed TGF-β1 (logTG-β1) was higher in patients with neuropathy than in those without LogTGFβ1 (OR7.5; P.006) [26]. TGF-β1 rs1982073C>T is 60.4% CC, 23.9% heterozygote CT, and 15.7% mutant homozygote TT. Allele analysis found 109 (27.7%) mutant T allele and 285 (72.3%) C allele.

Among Turkish osteoporotic patients and controls, Tural *et al*. reported the frequency of 18.4% and15.7% for CC genotype, 42.4% and 51.9% for CT genotype, and 31.6% and 22.2% for CC genotype, respectively [27]. Bivariate analysis found statistically significant difference where CT genotype is a protective factor of DFU (OR:0.28, 95%CI:0.13-0.60, p:0.001) compared to CC genotype. Multivariate analysis also found CT genotype in TGF-β1 rs1982073C>T is a protective factor of DFU compared to CC genotype. (OR: 0.29; 95%CI: 0.12-0.69, p: 0.005).

However, allele analysis shows no statistically significant difference found when comparing T allele with C allele (OR: 0.85, 95%CI: 0.55-1.33, p: 0.554). TGF-β1 rs1982073C>T is an SNP located in the first exon of the TGF β 1 gene. The C allele of TGF-β1 rs1982073 gene that codes proline has been associated with microvascular complication of DM, such as nephropathy, retinopathy and also neuropathy that is the main cause of DFU formation, when compared to T allele of TGF-β1 rs1982073 gene which codes leucine [17]. A study of 400 Caucasians with type-2 diabetes determined that rs1982073(C) was associated with diabetic nephropathy (OR: 1.85, 95%CI: 1.39-2.46, P<0.05) [27].

Although our allele analysis not enough to reach statistically significant association, the trend shows that T allele has smaller probability of DFU formation compared to C allele. (OR: 0.85, 95CI: 0.55-1.33).The genotypic distribution of the TGF-β1 rs1271283232T>C gene was 33.5% wild-type CC, 41.6% mutant heterozygote CT, and 24.9% mutant homozygote TT.

Allele distributions shows T as a mutant allele composed 180(45.7%) of total allele, and 214(54.3%) of C allele. Bivariate analysis show TT genotype of TGF-β1 rs1800469C>T is a risk factor of DFU compared to CC genotype (OR:2.3, 95%CI:1.11-5.06, p:0.025), And multivariate analysis shows TT genotype in rs1800469C>T is a risk factor of DFU (OR:3.18; 95%CI: 1.27-7.96, p:0.013).

Allele analysis shows T allele in TGF-β1 rs1800469 is a risk factor of DFU compared to C allele (OR: 1.65, 95%CI: 1.11-2.47, p:0.017).TGF-β1 rs1800469, also known as

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-509 C>T, is an SNP in the promoter region of TGF-β1 gene. This SNP does not change the nature of the TGF-β1 protein, instead it changes the amount of the protein produced. The T allele increases the amount of TGF-β1 produced, by preventing AP1 from binding to this region where the C allele would normally downregulate production. The increased TGF-β1 production with the T allele is thought to be the causes of all of the health effects of this SNP [29].The MMP9 gene in humans is located at chromosome 20q11.2-13.1. MMP9 rs367601348 located in coding sequence of MMP9 gene.[30] MMP9 rs367601348 is a missense varian in which

A allele codes Threonine (AAG), while G allele codes Alanine (GAG) although the phenotype effects of this polymorphism have not been observed yet [20]. The genotypic distribution of MMP9 rs367601348A>G gene was 17.3% wild-type AA, 48.2% mutant heterozygote AG, and 34.5% mutant homozygote GG. Allele distributions shows G as a mutant allele compose 231(58.6%) of total allele, and 163(41.4%) of C allele. while there is no significant difference found in MMP9 rs367601348A>G genes polymorphism. There is no statistically significant difference of allele comparison found for MMP9 rs367601348A>G.

This result suggests there might be no effects of the amino acid changes toward the MMP9 protein. MMP9 gene polymorphism is strongly thought to influence the process of ulcer formation and wound healing in patients with DFU. Increase in MMP9 is predicts wound healing in diabetic foot ulcer due to increase in extracellular matrix degradation and slows fibrosis [16, 17, 30]. MMP9 rs3918242C>T located at the upstream part of promoter and contains a binding site for transcriptional repression. In genotypes with the T allele, this transcriptional repression is reduced or even removed altogether, therefore potentially increasing the expression of MMP9 which cause an increase in extracellular matrix degradation, as well as slowing down interstitial fibrosis [30].

As a result, the healing and progression of ulcers become slower. On the other side, the C allele might potentially change the expression of MMP9 in favor of the promotion and healing of ulcers [19].

Previous study by K Singh (2013) found gene distribution in DFU (CC, TC, TT) of the MMP9 rs3918242C>T found it to be 54.6%, 42,7% and 2.7% [19]. Buraczynska *et al*. (2015) found gene distribution (CC, CT, TT) of the MMP9 rs3918242C>T of 62, 4%, 34, 2%, and 3, 4%, among their Caucasian study population [31].

T allele frequency in patients with DFU was 24.1% and in controls was 13.67%. Comparison of allele frequencies showed statistically significant difference between patients versus control group (OR: 2.112, 95% CI: 1.38-3.126, P: 00048 for DFU vs control). The combined risk genotype (CT + TT) frequencies of MMP9 rs3918242C>T (OR: 2.37, 95% CI: 1.47-4.81, p: 003 for DFU vs control). In this study frequency distribution of MMP9 rs3918242C>T is 51.8% wild-type CC, 45.7% mutant heterozygote CT, and 2.5% mutant homozygote TT.

Allele analysis found 294 (74.6%) mutant T allele, and 100(25.4%) C allele. Statistical analysis shows paradoxical results paradoxical results compared to the theory and previous studies. Bivariate analysis shows CT genotype of MMP9 rs3918242C>T found as a protective factor of DFU compared to CC genotype (OR:0.35, 95%CI:0.19-0.64, p:0.001), multivariate analysis shows CT genotype in MMP9 rs3918242C>T polymorphism is a protective factor of DFU compared to those with CC genotype (OR:0.36, 95%CI:0.19-0.69, p:0.002).

T allele distribution in MMP9 rs3918242 is a protective factor of DFU compared to C allele (OR: 0.41, 95%CI: 0.26-0.67, p: 0.001).Buraczynska *et al*. (2015) found that MMP9 rs3918242C>T polymorphism is significantly correlated with the risk of stroke in patients with and without DM, with T allele carriers were younger at the onset of stroke (63.5 ± 11.7 years) than patients with CC genotype (71 ± 14.1 years) (p <0.005) [31].

The results of both this study and ours provide insights on how single nuclear polymorphism of MMP9 gene produces changes in two different vascular beds of DM patients. As with existing studies, this study has several limitations, including the presence of other risk factors that could not be included in the analysis.

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However, it is hoped that this study can be used as a reference for genetic consultations of type-2 DM patients, and can lay down a foundation for further investigations.

Conclusion

In conclusion, CT genotype in TGF-β1 rs1982073, and CT genotype in MMP9 rs3918242 are the protective factor of DFU while TT genotype in TGF-β1 rs1800469 is a risk factor of DFU in diabetic patients compared to their wild type genotype. Allele analysis shows T allele mutation in TGF-β1 rs1800469C>T is a risk factor of DFU, while T allele mutation in MMP9 rs3918242C>T is a protective factor of DFU. These finding suggest these single nucleotide polymorphisms mentioned may have role in the DFU formation. Further studies needed to examine the effects of TGF-β1 rs1982073C>T, TGF-β1 rs1800469C>T and MMP9 rs3918242C>T to the changes of TGF-β1 and MMP9 cytokine expression and structure as the possible mechanism in which those polymorphism might affect the DFU formation process.

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