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Abstract **УДК 616.831-005.1-06:616.12-005.4:575.113.2(043.5)**

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THE ROLE OF VEGFA GENE POLYMORPHISM IN DIABETIC FOOT SYNDROME DEVELOPMENT

Introduction. The dysfunction of vascular endothelial growth factor A (VEGFA) is one of the leading factor of macro- and microangiopathy development in diabetic foot syndrome (DFS) patients. Recently, a number of experimental studies in various populations have been carried out to test the association between *VEGFA* gene polymorphisms and development of chronic hyperglycemia vascular complications. At the same time, there are no such studies in Ukrainian population.

Purpose. To check the possible association between C936T *VEGFA* gene polymorphism and DFS development in Ukrainian patients with type 2 diabetes mellitus (T2DM).

Materials and methods. Venous blood of 154 patients with T2DM complicated by DFS and 124 individuals without diabetes and glucose intolerance was used. The polymerase chain reaction-restriction fragment length polymorphism method (PCR-RFLP) was used for *VEGFA* C936T (rs3025039) polymorphism genotyping. Mathematical analysis of obtained data was performed using Statistical Package for Social Science software (SPSS, version 17.0, Chicago, IL, USA).

Results. It was found that ratio of C/C-homozygotes, C/T-heterozygotes and T/T-homozygotes (C936T *VEGFA* gene polymorphism) in patients with DFS was 47.4%, 41.6% and 11.0%; in the control group – 50.0%, 43.5%, 6.5%, respectively. Comparison of these genotypes frequencies between DFS patients and control subjects using χ^2-Pearson criterion showed no significant difference ($P = 0.413$). The results of regression analysis under the dominant, recessive, superdominant and additive inheritance models also revealed no association between *VEGFA* C936T genotypes and DFS development ($P > 0.05$).

Conclusion. There is no link between *VEGFA* C936T polymorphism and risk of DFS development in Ukrainian patients with T2DM.

Keywords: diabetic foot syndrome, gene polymorphism, VEGFA

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Резюме

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РОЛЬ ГЕНЕТИЧНОГО ПОЛІМОРФІЗМУ VEGFA У РОЗВИТКУ СИНДРОМУ ДІАБЕТИЧНОЇ СТОПИ

Порушення функціонування судинного ендотеліального фактору росту А (VEGFA) є однією із причин розвитку мікро- та макроангіопатій під час синдрому діабетичної стопи (СДС) у пацієнтів із цукровим діабетом. Останнім часом у різних популяціях світу виконана низка експериментальних робіт щодо вивчення зв'язку генетичного поліморфізму *VEGFA* із розвитком судинних ускладнень хронічної гіперглікемії. При цьому в українській популяції такі роботи відсутні. Метою даної роботи стало вивчення можливого зв'язку С936Т-поліморфізму гена *VEGFA* із розвитком СДС в українських пацієнтів із цукровим діабетом 2 типу (ЦД2).

У роботі була використана венозна кров 154 хворих з ЦД2, ускладненого СДС, і 124 осіб без цукрового діабету і порушень толерантності до глюкози. Для визначення поліморфізму С936Т (rs3025039) гена *VEGFA* була проведена полімеразна ланцюгова реакція з подальшим аналізом довжини рестрикційних фрагментів (PCR-RFLP). Статистичний аналіз був виконаний із використанням програми SPSS-17.

У результаті було встановлено, що у хворих з СДС співвідношення гомозигот СС, гетерозигот СТ і гомозигот ТТ за С936Т-сайтом гена *VEGFA* склало 47,4%, 41,6% і 11,0%, а в контрольній групі – відповідно 50,0%, 43,5%, 6,5%. Порівняння частот зазначених генотипів між пацієнтами з СДС і представниками групи контролю показало відсутність значущої різниці (P = 0,413). Результати регресійного аналізу також не встановили зв'язку генотипів за С936Т-локусом гена *VEGFA* із розвитком СДС (P > 0,05). Отже, в українських пацієнтів із ЦД2 відсутній зв'язок між С936Т-поліморфізмом гена *VEGFA* і ризиком розвитку СДС.

Ключові слова: синдром діабетичної стопи, генетичний поліморфізм, VEGFA.

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Introduction

Diabetes mellitus complicated by diabetic foot syndrome (DFS) is of significant relevance in highly developed countries of the world, as evidenced by the high level of fatal consequences, lower limb amputations and the place that DFS occupies among complex medical and social problems, requiring significant intellectual, organizational and economic resources [1, 2].

DFS was first separated as an independent disease by the WHO research group on diabetes mellitus in Geneva in 1987. This syndrome complicates the course of diabetes mellitus in 4–25% of patients. In such case, the risk of lower limb gangrene in patients is 20 times higher than in the general population. Almost every minute an amputation caused by DFS is performed somewhere in the world. The percentage of postoperative complications in such patients reaches 37, and postoperative mortality is 9–26% [3].

Identification of genetic factors of type 2 diabetes mellitus (DM2) is the most important part of research in the study of this pathology, since the identification of candidate genes for diabetes development allows to shed much light on the pathogenesis of the disease, the mechanisms of its complications, the principles of diagnosis, personalized treatment and prevention [4].

Endothelial dysfunction is known to play an important role in the pathogenesis of DFS. It is believed that one of the factors of occurrence of macro- and microvascular complications in DFS could be represented by a dysfunction of vascular endothelial growth factor A (VEGFA), which is a powerful mitogen of endothelial cells of blood vessels and protein, which ensures the migration of endo-

thelial cells, their invasion into the collagen gel and the formation of new blood vessels. In addition, VEGFA is necessary not only for the formation of normal vessels, but also for their maturation and survival [5]. The experiment shows that a long-term VEGFA concentration decrease or blockade leads to deterioration in the survival of endothelial cells, a decrease in the tissues of terminal arterioles and capillaries, and blood pressure increase [6].

The *VEGFA* gene is located on the short arm of chromosome 6 (6p21.3) and consists of 8 exons separated by 7 introns [7]. Today, according to ncbi website (https://www.ncbi.nlm.nih.gov/snp/?term=VEGFA), there are 4590 polymorphic sites in the *VEGFA* gene. One of the most clinically significant is the polymorphic locus C936T, which is located in the 3'-nontranslated area (3'UTR) of the gene. In different populations of the world it is shown that this polymorphic site is associated with the development of DM2 and its complications [8 – 10]. However, there is no data on the relationship of this single-nucleotide polymorphism with the development of DFS.

Objective. Study of potential link of C936T-polymorphism of the *VEGFA* gene with the development of DFS in Ukrainian patients with DM2.

Materials and methods. The blood of 154 patients with DM2 complicated by diabetic foot syndrome, who were hospitalized to MI "Sumy City Clinical Hospital No. 5" in the departments of vascular surgery and Department of Surgery No. 3 during 2011-2013, was used in the work. DFS was diagnosed on the basis of the Order of the Ministry of Health of Ukraine No. 1118 dated 21.12.2012 "On Approval and Implementation of Medical and Technological Documents on Standardization of Medical Care in Type 2 Diabetes Mellitus". Assessment of the condition of limbs included the detection of ulcers or amputations of the foot in history, symptoms of peripheral artery disease; detection of skin color changes in the lower extremities; detection of deformities of the foot and review of shoes, identifying visible signs of neuropathy, the initial stages of ischemia, nail deformation or damage. Neurological status was assessed in accordance with the standards of diabetic neuropathy assessment. Assessment of arterial blood flow included palpatory determination of its level and assessment of the symmetry of pulsation on the vessels of the lower extremities. Insufficiency of arterial blood flow in the lower limbs was assessed according to Fontaine-Leriche-Pokrovsky classification. Duplex ultrasound scanning of lower extremity arteries, rheovasography and angiographic examination were used as instrumental methods. Also, a bacteriological study of ulcerative exudate was conducted to determine the microflora and its sensitivity to antibacterial drugs. Laboratory studies included clinical analysis of blood and urine, determination of blood glucose, glycemic profile, glycosylated hemoglobin.

The control group consisted of 124 people without diabetes mellitus and impaired glucose tolerance. The absence of other multifactorial diseases was confirmed by collecting anamnestic data, electrocardiogram, blood pressure measurements, and biochemical studies. The study was conducted in compliance with the main provisions of the Council of Europe Convention on Human Rights and Biomedicine, the Helsinki Declaration of the World Medical Association on ethical principles of scientific medical research with human participation (1964, with subsequent amendments, including version as of 2000) and Order of the Ministry of Health of Ukraine No. 690 dated 23.09. 2009. Prior to venous blood sampling for genetic analysis all patients signed an informed consent. Blood sampling for genotyping was performed under sterile conditions into 2.7 ml monovettes (Sarstedt, Germany) containing potassium EDTA as anticoagulant. Samples were frozen and stored at -20 ºC.

DNA for genotyping was isolated from blood leukocytes using GeneJET Whole Blood Genomic DNA Purification Mini Kit (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. In order to determine C936T polymorphism (rs3025039) of the *VEGFA* gene a polymerase chain reaction with subsequent analysis of the restriction fragment length (PCR-RFLP) was performed. The *VEGFA* gene site containing polymorphic locus was amplified with a pair of specific primers: upstream (sense) – 5'-AAGGAAGAGGAGACTCTGCGCAGAGC-3' and downstream (antisense) – 5'-TAAATGTATGTATGTGGGTGGGTGTGTC-TACAGG-3'. Primers were synthesized by Metabion (Germany). For PCR, 50-100 ng of DNA was taken and added to a mixture, containing 5 µl of a 5-fold PCR buffer, 1.5 mmol/l of magnesium sulfate, 200 µmol/l of a mixture of four nucleotide triphosphates, 15 pmol/l of each primer and 1 unit of Taq-polymerase (ThermoFisher Scientific, USA), the volume was adjusted to 25 µl with deionized water. PCR was carried out in a thermal cycler GeneAmpPCR System 2700 (ThermoFisher

Scientific, USA). The amplification consisted of 33 cycles: denaturation – 94°C (50 s), hybridization – 59°C (60 s) and elongation – 72°C (60 s). For restriction analysis, 6 μl of the amplification product (208 pb) was incubated at 37°C for 18 h with the addition of 5 units of Hin1II restrictase (NlaIII) (ThermoFisher Scientific, USA). If +936th position of 3'-untranslated region of the *VEGFA* gene contained cytosine, endonuclease did not find restriction site and the original fragment 208 bp remained unchanged. In case of cytosine to thymine replacement, Hin1II restrictase split the amplificate into two fragments – 122 bp and 86 bp. Restriction fragments were separated by electrophoresis on 2.0% agarose gel containing ethidium bromide. After electrophoresis, DNA visualization was carried out using transiluminator "Biocom".

Statistical analysis was performed using SPSS-17 program. Continuous data are presented as the mean value ± SD (standard deviation), nominal data are presented as quantitative and percentage values. Testing of continuous data on the normality of distribution was carried out using the Kolmogorov-Smirnov test. Comparison of the distribution of genotypes in the experimental and control groups and verification of the correspondence of this distribution to the Hardy-Weinberg equilibrium was performed using Pearson's $\chi 2$-criterion. In order to establish the risk of DFS development, the odds ratio (OR) and 95% confidence interval (CI) for dominant, recessive, superdominant and additive inheritance models were calculated. Such DM2 risk factors, like age, gender, BMI, smoking, obesity and arterial hypertension (AH) were used as a covariate during multivariable logistic regression analysis. All tests were two-sided, value of $P < 0.05$ was considered statistically significant.

Study results. Clinical characteristics of 154 patients with DFS and 124 persons of the control group are presented in Table 1. No significant difference was found between the comparison groups in the indicators of mean diastolic blood pressure ($P = 0.109$), as well as in the ratio of smokers ($P = 0.173$), persons with BMI > 25 kg/m^2 ($P = 0.060$) and with AH ($P = 0.1121$). In this case, BMI ($P = 0.007$) and blood glucose concentration ($P < 0.001$) in patients were significantly higher compared to the control group ($P < 0.001$), and systolic blood pressure ($P < 0.001$), on contrary, was higher in patients without DFS. Also, the comparison groups differed in the ratio of persons of different sexes ($P = 0.038$) and the presence of persons with obesity ($P = 0.027$). In addition, it should be noted that the average age of the control group representatives (76.6 ± 10.2 years) was significantly higher than in patients with DFS ($P < 0.001$). The latter circumstance increased the reliability of control, because the probability of the development of DM2 and its complications in these people in the future decreased.

Table 1 – Characteristics of the patients with DFS and the controls

Parameter	**DFS (n = 154)**	**Control group (n = 124)**	**P**
Age, years	64.7 ± 8.2	76.6 ± 10.2	< 0.001
Sex, female/male	13/16	45/79	0.038
BMI, kg/m^2	29.3 ± 4.9	27.7 ± 4.9	0.007
Systolic pressure, mm Hg	9.99 ± 6.96	152.8 ± 23.2	< 0.001
Diastolic pressure, mm Hg	88.5 ± 9.6	86.4 ± 12.3	0.109
Fasting glucose, mmol/L	10.2 ± 3.5	5.29 ± 0.7	< 0.001
BMI > 25 kg/m^2, n (%)	122 (79.2)	86 (69.4)	0.060
Obesity, n (%)	59 (38.3)	32 (25.8)	0.027
Smokers, n (%)	50 (32.5)	31 (25.0)	0.173
Arterial hypertension, n (%)	108 (70.1)	76 (61.3)	0.121

Notes: n – number of patients, DFS – diabetic foot syndrome, BMI – body mass index. Categorical variables were compared by means of $\chi 2$-test, continuous variables were compared by means of t-test

Table 2 presents the frequencies of the main (C) and minor (T) alleles and shows the distribution of genotypes in C936T-polymorphic site 3'UTR of the *VEGFA* gene in patients with DFS and representatives of the control group. It is shown that the frequencies of these genotypes in the control and experimental groups did not deviate from the Hardy-Weinberg equilibrium (P > 0.05). Comparison of frequencies of three possible variants of genotypes, formed by polymorphic locus C936T of the *VEGFA* gene between patients with DFS and representatives of the control group showed no significant difference (P = 0.413).

Table 2 – Frequency of alleles and genotypes of C936T polymorphism in the *VEGFA* gene in the groups compared

	DFS (n = 154)	**Control group (n = 124)**
Homozygotes CC, n (%)	73 (47.4)	62 (50.0)
Heterozygotes CT, n (%)	64 (41.6)	54 (43.5)
Homozygotes TT, n (%)	17 (11.0)	8 (6.5)
C-allele	0.68	0.72
T-allele	0.32	0.28
χ^2	0.27	0.69
P	> 0.05	> 0.05

Notes: n – number of parients; DFS – diabetic foot syndrome; χ^2 and P reflect deviations from Hardy-Weinberg law in each group

The results of the regression analysis of the relationship of genotypes based on C936T-polymorphism of the *VEGFA* gene with the development of DFS in the framework of dominant, recessive, superdominant and additive inheritance models are shown in Table 3. The use of binary logistic regression did not reveal a reliable association of the studied locus with the development of DFS in any of the models (P > 0.05). After that, the multivariable logistic regression was used. However, even after correction for age, sex, BMI, obesity, hypertension and smoking habits, a significant association of different genotypes by polymorphic locus 3'UTR of the *VEGFA* gene with the development of DFS could not be established (P > 0.05).

Table 3 – Analysis of association between the C936T-polymorphism in the *VEGFA* gene and DFS in different inheritance models

Модель	**P_{obs}**	**OR_{obs} (95% CI)**	**P_{adj}**	**OR_{adj} (95% CI)**
Dominant	0.667	1.110 (0.691-1.781)	0.743	1.085 (0.667-1.765)
Recessive	0.189	1.799 (0.749-4.320)	0.218	1.761 (0.716-4.333)
Super-dominant	0.739	0.922 (0.571-1.487)	0.701	0.908 (0.556-1.484)
Additive*	0.979	1.007 (0.613-1.653)	0.962	0.988 (0.594-1.643)
	0.201	1.805 (0.729-4.466)	0.239	1.751 (0.690-4.447)

Note: 95% CI – 95% confidence interval; P_{obs} – observed P-value (not adjusted for covariates); OR_{obs} – observed odds ration; P_{adj} – observed P-value adjusted for age, sex, smoking status, BMI, obesity and AH status; OR_{adj} – odds ration adjusted for covariates.

[a]The first line in the additive model reflects the comparison of the CT-genotype with the CC-genotype, the second line reflects the comparison of the TT-genotype with the CC-genotype.

Discussion of the results. Single nucleotide polymorphism of rs3025039 is a replacement of cytosine for thymine at the +936th position of 3'-non-translated portion of the *VEGFA* gene. Such single point replacement may prevent binding of the transcription factor AP-4 (transcription factor activating enhancer binding protein 4), which in turn can affect the qualitative and quantitative characteristics of the future mRNA [11]. According to this, the relation of 936T-locus of the *VEGFA* gene with a decrease in its expression and plasma protein concentration has been demonstrated [11, 12].

In recent years, a number of works, dedicated to studying the effect of rs3025039-polymorphism of the *VEGFA* gene on the development of DM2 and its complications, were carried out. Thus, Sellami et al. demonstrated connection of this locus with the development and duration of DM2 among the Tunisian population [9]. A group of Russian authors, Klimontov et al., established a connection of C936T-site with VEGFA concentration in blood plasma and development of coronary heart disease in patients with DM2 [8]. And the results of the meta-analysis, conducted by Han et al., demonstrated a strong connection of C936T-polymorphism of the *VEGFA* gene with the risk of diabetic retinopathy among the Chinese population [10].

Our study was the first one to conduct the analysis of the relationship of C936T-polymorphic site of the *VEGFA* gene with the risk of DFS among the Ukrainian patients with DM2. Comparison of genotype frequencies at the specified locus between the patients of the experimental and control groups, as well as regression analysis with and without the correction for various risk factors of diabetes did not allow to establish link of C936T polymorphic site of the *VEGFA* gene with the development of DFS in the Ukrainian population.

However, the works of several teams have already proved the connection of other polymorphic loci of the *VEGFA* gene with the development of DFS. Xiaolei et al. demonstrated the protective role of rs699947-locus of the *VEGFA* gene regarding the DFS development in Chinese patients [13]. Li et al. found a similar protective effect regarding the DFS development, as well as a link with the protein content in blood plasma for the polymorphic site G-634C of the *VEGFA* gene [14]. Among the population of Iran, the link with the risk of DFS development for C-2578A polymorphism of of the *VEGFA* gene promoter was established [15].

It should be noted that a significant limitation of our study is the relatively small number of patients, included in the comparison groups. Thus, a possible association between the C936T-site and the risk of DFS could not be detected because of a small statistical force. Another limitation is the investigation of relationship between polymorphic locus and phenotype without assessing their effect on *VEGFA* mRNA content and its protein concentration in blood plasma.

Conclusions

Thus, the results of the study showed absence of relationship between C936T polymorphism of the VEGFA gene and the development of DFS among Ukrainian population. Both before and after correction for age, gender, smoking habit, body mass index, obesity, and hypertension, none of the genotypes were associated with the risk of DFS in patients with DM2.

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