

The role of NOS2A –954G/C and vascular endothelial growth factor +936C/T polymorphisms in type 2 diabetes mellitus and diabetic nonproliferative retinopathy risk management

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Abstract: Type 2 diabetes mellitus (T2DM) remains one of the major health problems in Europe. Retinopathy is one of the major causes of morbidity in T2DM, strongly influencing the evolution and prognosis of these patients. In the last 2 decades, several studies have been conducted to identify the possible genetic susceptibility factors involved in the pathogenesis of the disease. However, there is little data related to the involvement of vascular endothelial growth factor (VEGF) and nitric oxide synthase (NOS) gene polymorphisms in the T2DM Caucasian population. The objective of this study was to identify a possible connection between NOS2A –954G/C (rs2297518) and VEGF +936C/T (rs3025039) polymorphisms and the risk of developing T2DM and nonproliferative diabetic retinopathy in a Caucasian population group. We investigated 200 patients diagnosed with T2DM and 208 controls. Genotypes were determined by multiplex polymerase chain reaction-restriction fragment length polymorphism. Statistical and comparative analyses (Fisher's exact test) for dominant and recessive models of NOS2A –954G/C and VEGF +936C/T polymorphisms revealed an increased risk of T2DM ($\chi^2=8.14$, $\phi=0.141$, $P=0.004$, odds ratio [OR] =2.795, 95% confidence interval [CI] =1.347–5.801; $\chi^2=18.814$, $\phi=0.215$, $P<0.001$, OR =2.59, 95% CI =1.675–4.006, respectively). Also, comparative analysis for the recessive model (using Pearson's chi-square test [χ^2] and the phi coefficient [ϕ]) reveals that the variant CC genotype of NOS2A gene is more frequently associated with T2DM without retinopathy ($\chi^2=3.835$, $\phi=-0.138$, $P=0.05$, OR =0.447, 95% CI=0.197–1.015). In conclusion, the results of the study place VEGF +936C/T polymorphisms among the genetic risk factor for T2DM, whereas NOS2A –954G/C polymorphisms act like a protective individual factor for nonproliferative retinopathy.

Keywords: type 2 diabetes mellitus, T2DM, retinopathy, +936C/T variant of VEGF gene, –954G/C of NOS2A gene

Introduction

Type 2 diabetes mellitus (DM) is a major public health problem, being one of the most common metabolic disorders.^{1,2} Even if the hyperglycemic status has a hereditary component with dominant expression in the phenotype, diabetes is considered as a multifactorial disease in which there is an imbalance associated with hereditary and environmental factors.^{3,4} Over the last 2 decades, genome-wide association studies, linkage analysis, candidate gene approach, and combined analysis of these candidate loci led to the identification of several molecular markers associated with the pathogenesis of T2DM and its complications.^{5–7}

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Diabetic retinopathy (DR) is one of the major complications in diabetic adults and considered as the major cause of new-onset blindness in these patients. This microvascular complication occurs rapidly in some patients, whereas it occurs in the late stages of diabetic evolution or does not develop at all in other patients.^{8–10}

Vascular endothelial growth factor (VEGF) is a potent angiogenic and vascular permeability factor; therefore, this gene and its polymorphic variants seem to play an important role in DR characterized by impaired vascular permeability, tissue ischemia, and neoangiogenesis.^{11–13} The human *VEGF* gene is located on chromosome 6 (6p21.3) and highly polymorphic, with at least 30 single-nucleotide polymorphisms described in the literature.^{14–16} The particular +936C/T in the 3'-untranslated region of *VEGF* gene is one of the most common gene variants related to lower levels of plasma lipopolysaccharide-stimulated VEGF protein production in peripheral blood mononuclear cells in healthy individuals.^{17,18}

Human gene encoding for nitric oxide synthase (*NOS*) is located on chromosome 17 (17q11.2-12)¹⁹ and consists of a highly reactive intercellular signaling molecule, with anti-thrombogenic and antiplatelet regulatory activities, produced in the Muller cells and the retinal pigment epithelium²⁰ and highly expressed in retinal vessels, where it plays a major role in the regulation of vascular tone and macrophage remodeling.²¹ Although there is a sufficient evidence that endothelial NOS isoform is linked to diabetes and complications, such as retinopathy, inducible nitric oxide synthase (iNOS) remains unknown in terms of its association with DR. A pentanucleotide (CCTTT)*n* polymorphism was already proved to be associated with DR, although it is still questionable whether –954G/C polymorphism in the promoter region that affects the expression level of iNOS is also linked to DR.¹⁹

The objective of this study was to evaluate the possible association of +936C/T variant of *VEGF* gene and –954G/C of *NOS2A* gene in relationship with type 2 diabetes and nonproliferative retinopathy in a Caucasian of origin Eastern European population group.

Materials and methods

Patients and controls

The study was conducted according to the Declaration of Helsinki and was approved by the Ethics Committee of the “Iuliu Hatieganu” University of Medicine and Pharmacy, Cluj-Napoca, Romania. Written informed consent was obtained from all subjects included in this study.

A group of 408 individuals, all Caucasians, were included in this prospective case-control study; among those,

200 patients with T2DM were included in the study group without a medical history of high blood pressure or dyslipidemia (independent risk factor for retinopathy). For both groups, fasting blood glucose, total serum cholesterol, high-density lipoprotein and low-density lipoprotein cholesterol, triglyceride level, weight, body mass index, abdominal circumference, and systolic and diastolic blood pressure were determined. For the study group, glycosylated hemoglobin (HbA_{1c}) levels were determined in addition. Also, ophthalmological assessment with binocular indirect ophthalmoscopy and a standard fundus retinography (Visucam Lite; Carl Zeiss Meditec AG, Jena, Germany) was carried out in all diabetic subjects. We mention that in this study, only diabetic patients without retinopathy or with incipient, early-stage nonproliferative retinopathy were included. The controls consisted of 208 healthy, nondiabetic volunteers, with negative chronic ophthalmological illnesses.

Genotypic analyses of NOS2A –954G/C (rs2297518) and VEGF +936C/T (rs3025039)

DNA samples were obtained from 400 µL peripheral blood, using Wizard Genomic DNA Purification Commercial Kit (Promega Corporation, Fitchburg, WI, USA). Genotyping was based on polymerase chain reaction (PCR)-restriction fragment length polymorphism technique. For specific DNA amplification, a total amount of 100 ng of genomic purified DNA was amplified in a volume of 25 µL reaction mixture containing 12.5 µL of PCR mastermix, a premixed, ready-to-use solution containing Taq DNA polymerase, deoxynucleotide triphosphates, MgCl₂, and reaction buffers (Promega Corporation); 7.5 µL free nuclease water; 1 µL of bovine serum albumine; 1 µL of each primer; and 1 µL of water suspended DNA. The amplification products were submitted to enzyme digestion (Fermentas; Thermo Fisher Scientific, Waltham, MA, USA) and analyzed by electrophoresis agarose gel (MetaPhor®; FMC BioProducts, Rockland, ME, USA), allowing detection by ethidium bromide staining of the corresponding genotypes. Specific thermocycling conditions and resulting DNA fragments are presented in Table 1. The genotypic analyses of NOS2A –954G/C (rs2297518) and VEGF +936C/T (rs3025039) were done in accordance with the studies of Kun et al²² and Guan X et al.²³

Statistical analysis

Statistical analysis was carried out using SPSS 18.0 for Windows software (SPSS Inc., Chicago, IL, USA). Demographic and clinical data were compared using the Pearson's chi-square test (χ^2) and the phi coefficient. The distribution

Table 1 Specific thermocycling conditions and resulting DNA fragments

Gene variant	Primer sequence	Thermocycling conditions	Restriction enzyme	Fragments
NOS2A and -954G/C	F3'-CATATGTATGGGAATACTGTATTTTCAG-5' R5'-TCTGAACTAGTCACTTGAGG-3'	94°C/30 s, 61°C/45 s, 72°C/75 s, and 72°C/10 min	BsaI	GG (437 and 136 bp) GC (573, 437, and 136 bp) CC (573 bp)
VEGF and +936C/T	F3'-AAGGAAGAGGAGACTCTGCGCAGAGC-5' R5'-TAAATGTATGTATGTGGGTGGGTGTGTCTACAGG-3'	94°C/30 s, 64°C/1 min, 72°C/75 s, and 72°C/5 min	NlaIII	CC (208 bp) CT (208, 122, and 86 bp) TT (122 and 86 bp)

Abbreviations: NOS, nitric oxide synthase; F, forward; R, reverse; s, second; min, minute; VEGF, vascular endothelial growth factor.

of genotype and allele frequency of each *NOS2A* -954G/C (rs2297518) and *VEGF* +936C/T (rs3025039) polymorphisms between different groups was compared by the Fisher's exact test, followed by comparative analysis according to dominant and recessive models. For estimation of the relative risk and strength of association, we calculate odds ratio (OR) at 95% confidence interval (CI). A *P*-value <0.05 is considered to be statistically significant.

Results

Demographic characteristic and clinical data of the diabetic subjects and controls are presented in Table 2. The average disease duration was 7.2±5.4 years. Statistical analysis did not reveal any differences between subjects and controls as sex distribution ($\chi^2=1.370$, $\phi=0.058$, $P=0.242$), body mass index ($P=0.219$), weight ($P=0.943$), abdominal circumference ($P=0.731$), systolic blood pressure ($P=0.312$), and diastolic blood pressure ($P=0.341$). Although average

mean age in control group is lower compared with the study group ($P<0.001$), this has no influence on the genetic polymorphisms, because they are present in individuals, without being age related.

None of the subjects from the control group or the study group had high cholesterol or triglycerides level.

Genotype distribution and frequency of alleles of *NOS2A* -954G/C and *VEGF* +936C/T polymorphisms in patients with diabetes and controls are presented in Table 3.

Comparative analysis (Fisher's exact test) for dominant and recessive models of *NOS2A* -954G/C and *VEGF* +936C/T polymorphisms was performed.

Comparative analysis to assess the diabetes risk in the study group with that of the control group for -954G/C of *NOS2A* gene variant carriers (Fisher's exact test analysis – ORs for dominant model) does identify a statistically increased risk value ($\chi^2=8.14$, $\phi=0.141$, $P=0.004$, OR=2.795, 95% CI=1.347–5.801). The statistical analysis

Table 2 Demographic characteristics and clinical data of the diabetic and control subjects

	Diabetic subjects (n=200)	Control subjects (n=208)	OR	95% CI	P-value
Age	63.84±12.59	54.78±15	1.231	0.843–1.781	0.001
Fasting blood sugar	122.9±41.64	88.4±11	1.132	0.723–1.825	0.376
Weight (kg)	81.4±17.3	81.7±13.9	1.241	0.872–1.721	0.943
Body mass index (kg/m ²)	27.8±6.1	25.1±5.1	1.132	0.763–1.739	0.219
Abdominal circumference (cm)	90.4±11.8	87.9±10.3	1.192	0.812–1.769	0.731
Systolic blood pressure (mmHg)	124.51±15.35	120.71±17.25	1.251	0.629–1.871	0.312
Diastolic blood pressure (mmHg)	72.03±10.07	69.73±9.17	1.272	0.682–1.897	0.341
Disease's duration (years)	7.2±5.4	N/A	N/A	N/A	N/A
Cholesterol (mg/dL)	196.64±46.45	188.73±36.74	1.328	0.742–1.802	0.493
HDL (mg/dL)	46.14±10.07	49.04±11.71	1.287	0.620–1.891	0.529
LDL (mg/dL)	98.88±9.77	91.22±10.23	1.321	0.686–1.832	0.319
Triglyceride (mg/dL)	159.48±48.30	154.03±83.75	1.378	0.789–1.875	0.683
HbA _{1c}	6.95±0.8	N/A	N/A	N/A	N/A
Males, n (%)	101 (50.5)	93 (44.71)	1.262	0.855–1.862	0.275
Retinopathy, n (%)	123 (61.5)	N/A	N/A	N/A	N/A

Notes: Data shown as mean ± SD unless otherwise specified.

Abbreviations: OR, odds ratio; CI, confidence interval; SD, standard deviation; N/A, not applicable; HDL, high-density lipoprotein; LDL, low-density lipoprotein; HbA_{1c}, glycosylated hemoglobin.

Table 3 Genotype distribution and frequency of alleles in diabetic and control subjects

Polymorphisms	Variant	Diabetic subjects, n (%)	Control subjects, n (%)	OR (95% CI)	P-value
NOS	G/G	92 (46)	149 (71.63)	0.337 (0.223–0.508)	<0.001
	G/C	81 (40.5)	48 (23.08)	2.268 (1.477–3.483)	<0.001
	C/C	27 (13.5)	11 (5.29)	2.795 (1.346–5.801)	0.005
	G/C + C/C	108 (54)	59 (28.37)	2.964 (1.967–4.467)	<0.001
	G allele frequency	265 (66.25)	346 (83.17)	0.397 (0.285–0.552)	<0.001
	C allele frequency	135 (33.75)	70 (16.83)	2.518 (1.810–3.502)	<0.001
VEGF	C/C	118 (59)	164 (78.85)	0.386 (0.249–0.597)	<0.001
	C/T	60 (30)	38 (18.27)	1.917 (1.205–3.048)	0.007
	T/T	22 (11)	6 (2.88)	4.161 (1.650–10.492)	0.001
	C/T + T/T	82 (41)	44 (21.15)	2.590 (1.674–4.005)	<0.001
	C allele frequency	296 (74)	366 (87.98)	0.388 (0.268–0.563)	<0.001
	T allele frequency	104 (26)	50 (12.02)	2.571 (1.775–3.725)	<0.001

Abbreviations: OR, odds ratio; CI, confidence interval; NOS, nitric oxide synthase; VEGF, vascular endothelial growth factor.

according to Fisher's exact test – ORs for the recessive model – also reveals significant differences among patients and controls ($\chi^2=27.712$, $\phi=0.261$, $P<0.001$, OR =2.965, 95% CI =1.967–4.468).

Comparative analysis to evaluate the diabetes risk in the study group with that of the control group for +936C/T polymorphism of *VEGF* gene carriers (Fisher's exact test analysis – ORs for dominant model) does identify a statistically increased risk value ($\chi^2=18.814$, $\phi=0.215$, $P<0.001$, OR =2.59, 95% CI =1.675–4.006); the same statistically significant differences were highlighted by using Fisher's exact test – ORs for the recessive model ($\chi^2=10.506$, $\phi=0.160$, $P=0.001$, OR =4.161, 95% CI =1.650–10.493).

DR was also assessed by using the same Fisher's exact test – ORs. Comparative analysis for the dominant model to analyze DR in relation to the variant genotype for –954G/C of *NOS2A* gene did not reveal any statistically significant differences ($\chi^2=1.661$, $\phi=-0.091$, $P=0.197$, OR 0.685, 95% CI =0.385–1.219), whereas comparative analysis for the recessive model highlighted that the variant CC genotype is more frequently associated with diabetes without retinopathy ($\chi^2=3.835$, $\phi=-0.138$, $P=0.05$, OR =0.447, 95% CI =0.197–1.015).

Comparative analysis to analyze the diabetic non-proliferative retinopathy in relation to the variant genotype +936C/T of *VEGF* gene did not reveal any statistically significant differences for neither the dominant nor the recessive models ($\chi^2=0.215$, $\phi=0.033$, $P=0.643$ OR =1.147, 95% CI =0.642–2.052 and $\chi^2=1.316$, $\phi=0.081$, $P=0.251$, OR =1.769, 95% CI =0.661–4.739, respectively).

Nonproliferative DR assessed in relationship with HbA_{1c} , and variant genotypes of both investigated polymorphisms also found no statistical differences ($\chi^2=5.750$, $\phi=0.182$,

$P=0.016$, OR =2.171, 95% CI =1.146–4.113 for –954G/C of *NOS2A* variant and $\chi^2=5.288$, $\phi=0.143$, $P=0.021$, OR =3, 95% CI =1.158–7.772 for +936C/T of *VEGF* variant).

Discussion

Over the past 2 decades, the number of people diagnosed with T2DM has more than doubled globally, making it one of the major public health challenges to all developed countries. DR is the most common vascular complication of T2DM characterized by hemostatic abnormalities, increased vascular permeability, and tissue ischemia followed by neoangiogenesis, and it occurs in ~75% of patients within 15 years of evolution.²⁴ As this complication is one of the major causes of blindness in general population, the etiopathogenesis of this complication has been extensively studied in recent years.

However, only some of the diabetics develop this retinopathy, and this proves that there are a series of individual genetic factors involved in the long-term outcome of microvascular diabetic complications. Therefore, it is important to identify the genetic susceptibility factors for DR, which could help us to clarify the pathogenesis, evolution, and adopt adequate treatment.²⁵

The risk factors for DR include disease's length (period of time), fasting glucose, HbA_{1c} , and in addition high blood pressure, lipid serum levels, and genetic factors. The genetic risk factors remain a new, innovative field, requiring clear more scientific data that are involved in developing DR. We decided screening the subjects without high blood pressure and dyslipidemia and with a satisfactory glycemic control of the diabetes, especially for showing the role of genetic factors in DR.

Although *NOS2A* –954G/C and *VEGF* +936C/T polymorphisms and their relevance with diabetes and DR were studied

in several different studies^{4,5} in the last years, there are few data regarding the distribution and importance of these two genetic variants in Caucasian population with diabetes.

Genetic variants of VEGF with dysregulated expression are implicated in many chronic proliferative disorders, with markedly elevated protein levels in the vitreous and aqueous fluids in the eyes of patients with diabetes.²⁴ As VEGF may play an important role in the pathogenesis of DR, several polymorphisms have been studied in the *VEGF* gene. A meta-analysis found significant associations between +936C/T (rs3025039) and DR susceptibility in Asian populations;¹⁶ in accordance with other research group, a meta-analysis found that +936C/T polymorphism of *VEGF* gene was related to DR in Korean populations;²⁶ however, another study on Asian population demonstrated no significant association of this polymorphism with diabetes and associated retinopathy.²⁷ Our results come in agreement with these studies, placing +936C/T variant of *VEGF* gene among the genetic risk factors involved in the pathogenesis of type 2 diabetes and associated retinopathy.

Even if there are valid data regarding the distribution of this variant in the general population, it is still not clear whether there is a conclusive evidence for an association of this polymorphism with diabetes and retinopathy in East European Caucasian population.

It is well known that functional hyperemia in diabetic patients is severely decreased. The loss of this vascular response could starve the retina for oxygen and glucose, putting neurons at risk, and therefore contributing to retinal pathology.²⁸ Neuronal or glial retinal dysfunction observed in early stages of DR is associated with altered neurovascular signaling, leading to the loss of functional hyperemia. Because functional hyperemia is reduced and iNOS expression is increased, genetic polymorphisms of NOS2A gene are the suitable candidate molecular markers for the study of DR. Also, chronic inflammation has been proposed to be involved in the pathogenesis of obesity-related insulin resistance and type 2 diabetes,²⁹ and also a new study has shown that iNOS plays an important role in the pathogenesis of insulin resistance in vivo.³⁰ Another proof that iNOS may be involved in DR is that restoring functional hyperemia by inhibiting iNOS may slow the progression of the disease.²⁸

To our knowledge, there are no valid data regarding the assessment and relationship of -954G/C NOS gene polymorphism and diabetes in Caucasian population. -954G/C NOS was reported as a genetic risk variant in the inflammatory bowel disease in Spanish population or gastric cancer in the

Chinese population.¹⁹ The results of our study show that variant CC genotype of -954G/C NOS gene is associated with T2DM but is less commonly found in subjects with DR. Most of the late studies focused on the study of the pentanucleotide (CCTTT)*n* repeat polymorphism in *iNOS* gene, result suggesting that this particular genetic variant seems to act like a protective factor against retinopathy in patients with T2DM.^{19,30} Although our study is among the first case-control study focused on the association of NOS2A -954G/C and VEGF +936C/T genetic polymorphisms with the risk of T2DM and DR in the eastern European population, our study still has some limitations due to the relatively small sample size and lack of information regarding the evolution of retinopathy in these diabetic patients.

Conclusion

In this study, +936C/T polymorphism of *VEGF* gene is highly associated with DR; therefore, this genetic variant is confirmed to be an independent genetic risk factor for non-proliferative retinopathy. As for -954G/C polymorphism of *NOS* gene, there is evidence that the variant genotype acts like a protective factor against retinopathy in patients with type 2 diabetes.

Studied genetic polymorphisms may play an important role in genetic predisposition to DR. This finding suggests the need for interventional approaches to identify asymptomatic patients at risk of developing retinopathy, which can benefit from an aggressive prevention of DR.

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Disclosure

The authors report no conflicts of interest in this work.

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