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Endothelin-1 Gene Polymorphisms rs5370, rs1476046, and rs3087459 are not Associated with Diabetic Nephropathy in Caucasians with Type 2 Diabetes Mellitus

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Background: Diabetic nephropathy (DN) is a major microvascular complication of type 2 diabetes mellitus (T2DM). Several lines of evidence implicate the endothelin (ET) system in the pathophysiology of DN. The aim of the present study was to analyze if genetic polymorphisms of the ET-1 (*EDN1*) gene affect susceptibility to DN in Caucasians with T2DM.

Materials and methods: The study population consisted of 651 Caucasian subjects with T2DM of more than 10 years' duration: 276 patients with DN (cases) and 375 patients without evidence of DN (controls). Polymorphisms in ET-1 (*EDN1*) gene, rs5370, rs1476046, and rs3087459, were studied.

Results: Genotype distributions of the studied polymorphisms showed no significant difference between cases and controls.

Conclusions: We provide evidence that the rs5370, rs1476046, and rs3087459 polymorphisms of *EDN1* gene are not risk factors for DN in Caucasians with T2DM.

BACKGROUND

Diabetes mellitus type 2 (T2DM) is recognized as one the fastest growing chronic diseases. According to estimates its prevalence will increase worldwide from 371 million people in 2013 to 552 million people in 2030.[1] Diabetic nephropathy (DN) is a major microvascular complication of T2DM affecting up to 40% of patients with long-standing T2DM.[2] It is the leading cause of chronic kidney disease and end-stage renal disease and is associated with increased cardiovascular morbidity and mortality.[3]

DN is a complex multifactorial disease with a strong genetic component.[2] Familial aggregation studies have established that genetic susceptibil-

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ity plays a significant role in the development and progression of DN.[4] However, despite great efforts in identifying the causal genetic variants contributing to DN, results are quite inconsistent with multiple genes associated with a small effect in different populations.[5] In order to reduce the harmful effects of T2DM on renal function, it is of utmost importance to improve our understanding of the pathogenesis of DN, especially in relation to its genetic background.

The endothelin (ET) system consists of 3 endothelin isoforms (ET-1, ET-2 and ET-3), and two distinct, G-protein-coupled receptor subtypes designated ETA and ETB, linked to multiple signaling pathways.[6] Endothelin-1 (ET-1), a 21-amino acid peptide, is an intercellular signaling molecule expressed in many different organ systems and tissues.[6] While ET-1 is known mainly as one of the most potent vasoconstrictors, it also has mitogenic, prooxidative and proinflammatory properties that are especially relevant to the pathophysiology of diabetic vasculopathy.[7] In the kidneys, ET system plays a significant role in the paracrine/autocrine regulation of renal hemodynamics, salt and water homeostasis, and acid-base balance. In addition, it is essential in modulating renal cell proliferation, extracellular matrix accumulation, and inflammation.[6]

Multiple lines of evidence associate the ET system with the pathogenesis and progression of DN. T2DM activates the renal ET signaling pathway, which leads to progressive renal damage by cell proliferation, interstitial inflammation, fibrosis, and finally glomerulosclerosis.[8] Patients with T2DM have elevated systemic circulatory levels of ET-1, which correlate with markers of DN, such as hyperfiltration, mesangial expansion, and albuminuria.[9,10] Importantly, several studies showed that ET receptor antagonists ameliorate DN in experimental models[11,12] and the results were confirmed by subsequent clinical trials.[13-15]

ET-1 has also been shown to play an important role in the pathogenesis of diabetic retinopathy, another major microvascular complication of diabetes.[16] ET-1 plasma concentrations have been associated with the degree of progression of diabetic retinopathy.[17] Moreover, ocular tissues are a rich source of ET-1, which contributes to abnormal retinal hemodynamics in diabetic retinopathy.[18] Similar to findings in DN, endothelin antagonism has been shown to prevent diabetic retinopathy in animal models.[19]

The human ET-1 gene (*EDN1*) resides on chromosome 6p23-p24 region[20] and contains 5 exons and 4 introns distributed over 6.838 base pairs.[21] The mature ET-1 (amino acids 53-73) is encoded in exon 2.[21] The rs3087459 A>C and rs1476046 G>A polymorphisms are located in *EDN1* intronic regions, while the rs5370 G>T polymorphism is located in exon 5 region of *EDN1* and comprises an amino acid substitution (Lys → Asn) at codon 198 (http://www.ncbi.nlm.nih.gov/snp/).

EDN1 has been identified as a strong candidate gene for cardiovascular and renal disease. Various single nucleotide polymorphisms (SNPs) in the *EDN1* gene were linked to arterial hypertension[22,23], heart failure[24], coronary artery disease[25,26], left ventricular hypertrophy[27], modification of vascular reactivity[28], endothelial dysfunction[29], progression of atherosclerosis[30], as well as with accelerated decline in kidney function in the chronic glomerulonephritis patients[31], and with end stage renal disease attributed to hypertension[32]. In addition, *EDN1* genetic variability was related to microvascular complications of T2DM such as diabetic retinopathy[33], and DN[34]. However, the results of the association studies published so far are inconsistent.[35,36]

According to the aforementioned evidence, *EDN1* gene is a robust biological candidate as a susceptibility locus for DN in subjects with T2DM. The aim of the present study was to clarify whether common single nucleotide polymorphisms of the *EDN1* gene (rs3087459, rs1476046, rs5370) are associated with DN in subjects with T2DM in Caucasian population.

MATERIALS AND METHODS

In this cross-sectional case-control study we enrolled 651 unrelated Caucasians with T2DM of more than 10-year duration from outpatient clinics of the University Medical Centre Maribor and General Hospitals Murska Sobota and Slovenj Gradec. The study group consisted of 276 subjects with DN (cases) and 375 subjects without clinical signs of DN (control group). Patients were classified as having T2DM according to the current American Diabetes Association criteria.[37] Diagnosis of DN was made according to World Health Organization 1999 diagnostic criteria.[38]

To avoid the confounding effect of impaired kidney function, patients with overt nephropathy were not enrolled in the study. Patients with poor glycaemic control, significant heart failure (NYHA II-IV), alcoholism, infection, and other causes of renal disease were also excluded. The study was

approved by the national medical ethics committee and was performed in compliance with the Helsinki declaration. After an informed consent for the participation in the study was obtained, a detailed interview was conducted. Information on smoking, presence and family history of cardio-vascular disease (CVD), duration of arterial hypertension and T2DM, T2DM management and complications (retinopathy, neuropathy, and diabetic foot), therapy and routine laboratory measurements were obtained from patients' medical records.

Biochemical analyses

Total cholesterol, low-density lipoproteins (LDL), high-density lipoproteins (HDL), triglycerides (TG), cystatin C, fasting glucose, hemoglobin (Hb), glycated hemoglobin (HbA1c), urea, and creatinine were determined in serum by standard biochemical methods. For each patient, albumin-to-creatinine ratio was determined in three urine samples, according to diagnostic criteria. Cystatin C and MDRD study equation were used to estimate glomerular filtration rate (eGFR).

Genotyping

Genomic DNA was extracted from 100 μl of whole blood using a Qiagen isolation kit. The rs5370, rs1476046, and rs3087459 polymorphisms of the *EDN1* gene were genotyped by KBioscience Ltd using their own novel fluorescence-based competitive allele-specific PCR (KASPar) assay. Details of the method used can be found at http://www.kbioscience.co.uk/.

Statistical analysis

Statistical analyses were conducted with the use of the SPSS program for Windows version 19 (SPSS Inc. Illinois). Continuous clinical data were compared by unpaired Student's *t*-test, while chi-square test was used to compare discrete variables. Data were expressed as mean ± SD (continuous variables) or as the number and percent of patients (categorical variables). Further, all variables that showed significant differences by univariate analysis (with a p value < 0.05 considered significant) were analyzed together in a logistic regression analysis. A $p < 0.05$ was considered statistically significant. The deviation from Hardy-Weinberg equilibrium (HWE) was assessed by the exact test (http://ihg.gsf.de/).

RESULTS

The demographic and clinical characteristics of the cases and control subjects are listed in **Table 1**. There were no significant differences between groups with respect to age, sex, duration of T2DM, diastolic blood pressure, body mass index (BMI), smoking status, duration of diabetic retinopathy, eGFR, Hb, and total cholesterol, HDL, and LDL cholesterol levels. On the other hand, statistically significant differences were observed in the following parameters: duration of hypertension, systolic blood pressure, family history of cardio-vascular disease (CVD), urine albumin/creatinine ratio, HbA1c, TG, fasting glucose, urea, and creatinine levels. Cases also showed significantly more other chronic diabetic complications (diabetic retinopathy, diabetic foot).

Differences in parameters reflecting renal function (creatinine, cystatin C, eGFR and urine albumin/creatinine ratio) confirmed chronic kidney disease in diabetic subjects with DN. Cystatin C was a better marker for estimation of renal function than eGFR (MDRD equation ml/min). Cystatin C was significantly higher in subjects with DN ($p<0.001$) **(Table 1)**.

The genotype distribution and allele frequencies of *EDN1* gene rs5370, rs1476046, and rs3087459 polymorphisms in subjects with DN (cases) and in those without DN (controls) are presented in **Table 2**. Univariate analysis didn't reveal significant differences in the genotype or allele frequencies between T2DM cases and controls **(Table 2)**. The genotype distribution didn't significantly deviate from Hardy-Weinberg equilibrium **(Table 2)**.

Similarly, no association between the studied polymorphisms and DN was found in a multivariate logistic regression analysis adjusted for different confounders (duration of hypertension, systolic blood pressure, CVD, diabetic retinopathy, diabetic foot, HbA1c, fasting glucose, urea, creatinine, cystatin C, urine albumin/creatinine ratio) according to co-dominant genetic model.

DISCUSSION

In the present case-control study that included 651 Caucasian subjects with T2DM, we failed to confirm an association between the rs5370, rs1476046, and rs3087459 polymorphisms of the *EDN1* gene and DN. The minor allele frequencies (MAF) of the three *EDN1* polymorphisms in our T2DM sample were comparable to global MAF obtained from SNP database (dbSNP).[39]

The kidneys are extremely sensitive to ET-1 and the components of ET system are present throughout the nephron and renal vasculature.[40] ET-1 is produced by glomerular epithelial and mesangial

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Table 1. Clinical and laboratory characteristics of cases and controls

	Cases (DN+)	Controls (DN-)	Sig. (p)
No.	276	375	
Sex (M)	59.1%	52.4%	0.1
Age (years)	64.75±9.15	63.75±8.0	0.13
Duration of T2D (years)	14.71±7.97	14.60±6.73	0.84
Duration of hypertension (years)	12.23±9.88	10.52±8.22	0.02
SBP [mm Hg]	155.27±18.92	149.84±19.63	<0.001
DBP [mm Hg]	84.87±11.63	84.06±11.42	0.36
BMI	31.3±4.68	30.77±5.0	0.23
Active smokers	6.6%	8.9%	0.31
CVD	20.0%	12.2%	0.007
Family history of CVD	41.3%	58.7%	0.91
DR	37.8%	24.6%	<0.001
Duration of DR (years)	3.94±3.11	6.54±7.03	0.23
DNeur	9.1%	6.0%	0.38
DF	15.5%	8.1%	0.03
S-HbA1c [%][1]	7.98±1.38	7.65±1.14	0.001
S-fasting glucose [mmol/l]	9.03±2.76	8.51±2.53	0.01
S-Hb [g/l]	139.39±14.91	139.40±12.96	0.99
S-urea [mmol/l]	7.35±3.73	6.25±1.91	<0.001
S-creatinine [μmol/l]	93.13±58.21	78.44±20.15	<0.001
male sex	101.57±61.84*	84.28±19.94*	<0.001*
female sex	79.7±49.21**	71.91±18.35**	**<**0.001
eGFR [MDRD equation, ml/min]	72.6±19.74	75.22±15.16	0.22
male sex	71.97±19.45*	77.66±14.33*	0.002*
female sex	74.31±20.72**	72.45±15.69**	0.13**
S-cystatin C [mg/l]	0.95±0.48	0.78±0.21	**<**0.001
S-Total cholesterol [mmol/l]	4.62 ± 1.17	4.55 ± 0.99	0.42
S-HDL [mmol/l]	1.23 ± 0.35	1.26 ± 0.36	0.29
S-LDL [mmol/l]	2.59 ± 0.95	2.57 ± 0.80	0.73
S-TG [mmol/l]	2.08 ± 1.6	1.83 ± 1.24	0.04
U-albumin/creatinine ratio [g/mol] - sample No. 1	27.49 ± 55.46	1.57 ± 3.05	<0.001
U-albumin/creatinine ratio [g/mol] - sample No. 2	23.13 ± 39.34	1.60 ± 3.67	<0.001
U-albumin/creatinine ratio [g/mol] – sample No. 3	23.36 ± 42.49	1.62 ± 2.49	<0.001

The values represent mean ± standard deviation. Bold indicates statistically significant results.
[1] The average value for hemoglobin A1c (HbA1c).
* comparing eGFR in men with DN versus men without DN
** comparing women with DN versus women without DN

cells, podocytes, renal tubular and medullary collecting duct cells, but probably also by local and infiltrating macrophages.[41] ETA receptors promote vasoconstriction, cell proliferation, fibrosis, and podocyte damage; while ETB receptors mediate vasodilator, anti-inflammatory, antiproliferative and antifibrotic effects.[41] Accordingly, ET-1 is thought of particular importance in the progression of chronic kidney disease.

The ET system is chronically activated in dia-

Table 2. Distribution of ET-1 (EDN1) gene rs5370, rs1476046, and rs3087459 polymorphisms genotypes and alleles in patients with diabetic nephropathy (cases) and in those without diabetic nephropathy (controls)

	Cases (276)	Controls (375)	p value
Rs3087459			
CC	6 (2.2)	13 (3.4)	0.6
AC	81 (29.3)	110 (29.3)	
AA	189 (68.5)	252 (67.3)	
C allele (%)	93 (16.8)	136 (18.1)	
A allele (%)	459 (83.2)	614 (81.9)	0.6
PHWE†	0.4	0.8	
Rs5370			
TT	12 (4.3)	22 (5.9)	0.4
TG	116 (42.1)	139 (37.1)	
GG	148 (53.6)	214 (57.0)	
T allele (%)	140 (25.4)	183 (24.4)	
G allele (%)	412 (74.6)	567 (75.6)	0.7
PHWE†	0.07	0.9	
Rs1476046			
AA	12 (4.3)	28 (7.5)	0.1
GA	116 (42.1)	137 (36.5)	
GG	148 (53.6)	210 (56.0)	
A allele (%)	140 (25.4)	193 (25.7)	
G allele (%)	412 (74.6)	557 (74.3)	0.9
PHWE†	0.07	0.4	

PHWE† values were computed using Pearson's goodness-of-fit chi-square (1 df)

Table 3. Multivariate logistic regression analysis adjusted for different confounders (duration of hypertension, systolic blood pressure, CVD, diabetic retinopathy, diabetic foot, HbA1c, fasting glucose, urea, creatinine, cystatin C, urine albumin/creatinine ratio) according to co-dominant genetic model

Inheritance Model	Genotype	Cases (276)	Controls (375)	Adjusted OR, 95% CI/p†-value
Rs3087459	CC	6 (2.2)	13 (3.4)	0.66 (0.10 – 1.02)/0.7
Co-dominant	AC	81 (29.3)	110 (29.3)	0.75 (0.39 – 1.45)/0.4
	AA	189 (68.5)	252 (67.3)	Reference
Rs5370	TT	12 (4.3)	22 (5.9)	2.26 (1.28 – 2.37)/0.2
Co-dominant	TG	116 (42.1)	139 (37.1)	2.78 (1.26 – 2.96)/0.4
	GG	148 (53.6)	214 (57.0)	Reference
Rs1476046	AA	12 (4.3)	28 (7.5)	0.34 (0.27 – 0.65)/0.2
Co-dominant	GA	116 (42.1)	137 (36.5)	0.53 (0.51 – 1.58)/0.6
	GG	148 (53.6)	210 (56.0)	Reference

P† values were adjusted for duration of hypertension, SBP, CVD, DR, DF, haemoglobin A1C (HbA1c), S-fasting glucose, S-urea, S-creatinine, S-cystatin, U-albumin/creatinine ratio [g/mol] - sample No. 1-No.3.

Odds ratio (OR); Confidence interval (CI).

betic patients with DN as evidenced by elevated circulating levels of ET-1[9], increased kidney ET-1 concentrations[42], and increased renal and systemic ETA receptor activation[43]. Stimulation of ETA receptors at the glomerular level promotes mesangial cells proliferation and podocyte injury, resulting in proteinuria and glomerulosclerosis.[41] Further, chronic inflammation plays a key role in the development and progression of DN and ET-1 has been shown to exert a number of pro-inflammatory effects.[41] It acts as a chemo-attractant for neutrophils[44] and monocytes[45], and activates the endothelium to increase leucocyte adhesion and transmigration[46], thus increasing leukocyte infiltration within the kidney. Overexpression of human ET-1 in the kidney of transgenic mice induced prominent renal fibrosis that resulted in progressive decrease in glomerular filtration rate leading to fatal kidney disease.[47] In contrast, blockade of ET-1 signaling revealed renoprotective effects in experimental models of DN, independent of blood pressure reduction[12], with a decrease in proteinuria, and evidence of reduced glomerulosclerosis.[11] In addition, anti-inflammatory effects of ET receptor antagonists resulted in reduced expression of intercellular adhesion molecule 1 and MCP-1[48], as well as reduced renal monocyte infiltration and expression of proinflammatory cytokines in different models of DN[49,50]. Atrasentan, a selective ETA receptor antagonist, was efficacious in lowering residual albuminuria in subjects with T2DM on stable doses of RAS inhibitors.[14,15]

EDN1 gene represents a strong candidate as a susceptibility locus for the manifestations and progression of diabetic macro- and microvascular complications, including DN. Of the studied polymorphisms, the rs5730 polymorphic variant was associated with blood pressure in overweight people of European[22] and Japanese descent[23]. Iglarz et al. confirmed the impact of rs5370 polymorphism on vascular reactivity[28], while Treiber et al. demonstrated that the rs5370 polymorphism influences blood pressure response to behavioral stress in obese subjects from lower socio-economic status[51]. Li et al. identified the Asn/Asn genotype of *EDN1* rs5370 polymorphism as a genetic factor for delayed onset of T2DM and reduced risk of diabetic retinopathy in T2DM patients of Chinese descent.[33] The rs5370 polymorphism in exon 5 region of EDN1 results in Lys/Asn amino acid substitution at codon 198, which may affect the processing of pre-proET-1 to mature ET-1.[22] While the functional relevance of the rs5370 polymorphism is as yet unknown, it was associated with elevated levels of plasma C-terminal-pro-endothelin-1, a biologically stable surrogate of ET-1, in people of European ancestry.[52]

The functional significance of rs3087459 and rs1476046 polymorphisms, which are located in EDN1 intronic regions, is also not clear. Intriguingly, in Chinese subjects with T2DM the EDN1 gene intron variant rs1476046 was associated with both plasma C-terminal pro-endothelin-1 concentrations and with DN.[34] In addition, rs1476046 was associated with endothelial dysfunction in pre-pubertal children, particularly in the presence of obesity.[29] While the rs3087459 polymorphism was studied in patients with coronary artery disease[26], there are no reports of associations with T2DM or DN.

The discrepancy between the results of our and other genetic association studies may be partly explained by differences in phenotype definition, the variation in the genetic or environmental background of the populations studied, or by insufficient sample size.[53] While the number of subjects included in our study was relatively small, they were recruited form a rather homogenous genetic and environmental background. In addition, the strength of the study is a rather long duration of T2DM in both cases and control subjects. Regrettably, we didn't measure the plasma levels of ET-1 or C-terminal-pro-ET-1.

CONCLUSION

In conclusion, we did not find an association between either the rs5370, or rs1476046, or rs3087459 polymorphisms of the EDN1 gene and DN in Caucasians with T2DM, indicating that they are not genetic markers for susceptibility to DN in Caucasians with T2DM.

CONFLICT OF INTEREST

All authors declare no conflict of interest.

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Полиморфизмы гена эндотелина-1 rs5370, rs1476046, и rs3087459 не связаны с развитием диабетической нефропатии среди представителей белой расы с сахарным диабетом 2-ого типа

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Введение: Диабетическая нефропатия (ДН) является основным микрососудистым осложнением захарного диабета 2-ого типа (СД2). Существует несколько доказательств, связывающих эндотелиновую систему (ЭТ) с патофизиологией ДН. Целью настоящего исследования является анализ возможного воздействия полиморфизмов гена ЭТ – 1 (EDN1) на предрасположенность к ДН представителей белой расы с СД2.

Методы: Исследование было проведено среди 651 субъекта белой расы с СД2 на протяжении более 10 лет: 276 пациентов с ДН (случаи) и 375 пациентов без наличия ДН (контрольная группа). Были исследованы полиморфизмы гена ЭТ - 1 (EDN1) rs5370, rs1476046, и rs3087459.

Результаты: Генотипическое распределение исследованных полиморфизмов не установило существенных различий между случаями и контрольной группой.

Заключение: Нами представлены доказательства, что rs5370, rs1476046, и rs3087459 полиморфизмы гена EDN1 не являются факторами риска развития ДН среди представителей белой расы с СД2.