Original Paper

***NOS3* 894G>T Polymorphism is Associated With Progression of Kidney Disease and Cardiovascular Morbidity in Type 2 Diabetic Patients: *NOS3* as a Modifier Gene for Diabetic Nephropathy?**

Katarína Kuricová^{a,c} Veronika Tanhäuserová^{a,c} Lukáš Pácal^a Vendula Bartáková^a
Lucie Brožová^b Jiří Jarkovský^b Kateřina Kaňková^a

^aDepartment of Pathophysiology, Faculty of Medicine, Masaryk University; ^bInstitute of Biostatistics and Analyses, Masaryk University, Brno, Czech Republic; ^cBoth authors contributed equally to this work

Key Words

Diabetic nephropathy • Nitric oxide synthase • Polymorphism • Cardiovascular morbidity

Abstract

Background/Aims: We have previously associated SNP 894G>T in the *NOS3* gene with diabetic nephropathy (DN) using multi-locus analysis. Variant 894G>T has been widely studied as a DN susceptibility factor with contradictory results. In the present study we genotyped 894G>T in the cohort of prospectively followed type 2 diabetics with the aim to investigate its possible role in the progression of DN and development of morbidity and mortality associated with diabetes. **Methods:** 311 subjects with defined stage of DN were enrolled in the study and followed up for a median of 38 months. We considered three end-points: progression of DN, major cardiovascular event and all-cause mortality. **Results:** Considering baseline GFR, age at enrolment and diabetes duration as confounders, Cox regression analysis identified 894GT genotype as a risk factor for DN progression (HR = 1.843 [95% CI 1.088 – 3.119], P = 0.023) and 894TT genotype as a risk factor for major cardiovascular event (HR = 2.515 [95% CI 1.060 – 5.965], P = 0.036). **Conclusion:** We ascertained the significant effect of the *NOS3* 894G>T variant on DN progression and occurrence of major cardiovascular event in T2DM subjects. Based on these results *NOS3* can be considered a modifier gene for DN.

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Introduction

Chronic hyperglycaemia accompanying diabetes is responsible for the development of diabetes-specific microvascular pathologies of which diabetic nephropathy (DN) is a

common and serious complication of diabetes since, at certain point, renal function starts declining, i.e. chronic kidney disease (CKD) eventually progresses to the end-stage renal disease (ESRD). Both diabetes itself and associated CKD increase cardiovascular morbidity and mortality making diabetes one of the most common causes of death worldwide [1].

Contribution of genetic factors to the development of DN has been soundly established. The evidence was initially provided by studies showing familial clustering [2-4], differences between ethnic groups [5] and segregation analyses [6, 7]. Subsequently, numerous genetic association studies were carried out, typically using candidate gene approach and focusing on a single gene at the time. Before the onset of genome-wide association studies (GWAS) we attempted to take into account likely polygenic pattern of DN genetic susceptibility and employed the multi-locus approach using the set-association analysis in a case – control study comprising 419 type 2 diabetics and 228 healthy subjects genotyped for 45 single nucleotide polymorphisms (SNPs) in 20 candidate genes for DN [8]. Using such approach we identified SNPs in four genes – *AGER*, *EDN1* and *LTA* on chromosome 6p and *NOS3* on chromosome 7q - associated with DN in type 2 diabetes (T2DM).

So far only few GWAS in DN were performed. The first one, performed on a Japanese population with type 1 diabetes mellitus (T1DM), identified association of SNP in *SLC12A3* with DN [9]. Next GWAS performed on Pima Indians with T2DM revealed *PVT1* as a susceptibility gene for ESRD in diabetes [10]. Results of the first GWAS in T1DM subjects with European descent have been published in 2009 [11]. The study identified SNPs in several genes as possibly contributing to ESRD susceptibility however these associations did not reach genome-wide significance. Another study on European T1DM population found association of SNPs in *FRMD3* and *CARS* with DN [12]. First GWAS in African Americans with T2DM identified several SNPs as potential candidates, however, genome-wide significance has not been reached [13]. Recently, a large meta-analysis of GWAS of DN in T1DM detected association of the three new SNPs with ESRD and DN [14]. Altogether, results of currently available GWAS are inconsistent with only several SNPs associated with DN susceptibility (and typically not shared by different populations or diabetes types).

Endothelial isoform of the nitric oxide synthase, encoded by *NOS3* catalysing production of nitric oxide is involved in the regulation of vascular tone and large body of data supports its role in the pathogenesis of DN [15]. Due to the above mentioned (pathogenically plausible) association of the *NOS3* with DN we were interested whether our previous finding of association with the susceptibility to DN using cross-sectional study design could be extended to its role in the modulation of DN progression, i.e. the role of the *NOS3* as a modifier gene. Therefore, the aim of the study was to replicate genotyping of *NOS3* 894G>T SNP in an independent cohort of prospectively followed T2DM subjects with variable stage of DN at baseline to ascertain its possible predictive potential for the progression of DN (primary outcome), cardiovascular morbidity and mortality and all-cause mortality (secondary outcomes).

Materials and Methods

Subjects

A total of 311 consecutive unrelated T2DM subjects (157 men and 154 women) with variable stage of kidney damage followed in Diabetes and Nephrology units of the two University hospitals in Brno, Czech Republic were enrolled into the study between 2002 and 2007. Prospective data were collected until the end of 2011. The stage of DN was defined according to the urinary albumin excretion (UAE). At baseline our study sample consisted of: normoalbuminuric subjects (UAE < 30 mg/24 h, 9.6 %), microalbuminuric subjects (UAE 30–300 mg/24 h, 35.7 %), macroalbuminuric subjects (UAE > 300 mg/24 h, 42.1 %) and subject with end-stage renal disease (ESRD, = CKD V, 12.6 %) for whom UAE was not ascertained. Stage of CKD was determined by glomerular filtration rate (GFR) assessed by creatinine clearance based on 24 h urine collection. Respective staging for CKD in the same sample was: CKD I (GFR ≥ 90 ml/min per 1.73 m², 19.6 %), CKD II (60–89 ml/min per 1.73 m², 18.2 %), CKD III (30–59 ml/min per 1.73 m², 32.3 %), CKD IV

Table 1. Baseline demographic and clinical characteristics of the subjects

Parameter (unit)	Normoalbuminuria (n = 30)	Microalbuminuria (n = 111)	Macroalbuminuria (n = 131)	ESRD (n = 39)	P
Age (years)	64 [59 – 72]	68 [57 – 75]	67 [61 – 74]	72 [62 – 78]	NS
Duration of diabetes (years)	13 [8 – 16]	10 [6 – 18]	16 [8 – 21]	17 [14 – 22]	0.002
FPG (mmol/l)	7.7 [6.2 – 10.0]	8.1 [7.2 – 11.2]	8.9 [7.0 – 11.1]	8.4 [6.2 – 10.2]	NS
HbA _{1c} (%)	6.4 [5.2 – 7.5]	6.5 [5.3 – 8.1]	7.7 [5.7 – 8.8]	6.2 [5.2 – 7.2]	NS
Triglycerides (mmol/l)	1.9 [1.4 – 2.8]	2.0 [1.4 – 3.0]	2.1 [1.5 – 3.5]	2.0 [1.6 – 2.5]	NS
Total cholesterol (mmol/l)	5.0 [4.3 – 6.2]	4.9 [4.3 – 5.7]	5.1 [4.3 – 6.1]	4.5 [3.6 – 5.4]	0.027
Creatinine (μmol/l)	88 [81 – 105]	115 [91 – 153]	162 [123 – 237]	524 [462 – 632]	<0.001
Proteinuria (g/24 h)	0.11 [0.09 – 0.12]	0.13 [0.08 – 0.21]	1.55 [0.60 – 3.25]	-	<0.001
GFR (ml/min per 1.73 m ²)	95.5 [79.3 – 138.0]	57.5 [41.6 – 86.0]	45.4 [29.4 – 70.1]	-	<0.001
UAE (mg/24 h)	11 [8 – 13]	60 [26 – 160]	404 [111 – 752]	-	<0.001

Data are expressed as median [interquartile range]. Comparisons made by Kruskal-Wallis ANOVA.

Abbreviations: ESRD, end stage renal disease; FPG, fasting plasma glucose; GFR, glomerular filtration rate; HbA_{1c}, glycated haemoglobin; UAE, urinary albumin excretion

(15–29 ml/min per 1.73 m², 13.4 %) and subjects with CKD V at baseline (GFR < 15 ml/min per 1.73 m² or maintenance haemodialysis, 16.5 %). Both parameters – UAE and GFR – were repeatedly measured at least once in 6 months or more often, staging for DN and CKD was based on two consecutive values. Retinopathy at baseline was confirmed in 35.8 % of subject and neuropathy in 28.4 %. Treatment of diabetes consisted of oral anti-diabetic drugs (52.3 % of subjects) and insulin (55.4 %), renoprotective treatment of angiotensin-converting enzyme inhibitors (63.5 %) and angiotensin II type 2 receptor blockers (42.2 %). Clinical characteristics of patients are shown in Table 1. All subjects originated from the South Moravia region of Czech Republic and all were of the Caucasian origin. Informed consent was obtained from each patient prior to being included in the study. The study was performed in accordance with principles of the Declaration of Helsinki and approved by the Ethical committee of the Faculty of Medicine, Masaryk University Brno.

Follow-up data

Subjects were followed for a median of 38 [21 – 65] months. Three end-points were considered: (i) progression of DN (i.e. transition from any given baseline DN stage except of ESRD at baseline to a more advanced stage of albuminuria/proteinuria or to ESRD), (ii) major cardiovascular event (non-fatal or fatal myocardial infarction or stroke, limb amputation, revascularisation) and (iii) all-cause mortality. In case of the assessment of the progression of DN (primary outcome), patients with ESRD at baseline (n = 39) were excluded from this analysis since they could not progress further.

Detection of polymorphism

Sample of peripheral venous blood was taken from each subject at the time of enrolment in the study. DNA was extracted from separated peripheral blood leukocytes by the phenol-chloroform method and stored at -20°C until further analysis. NOS3 894G>T (E298D, rs1799983) SNP was detected using polymerase chain reaction with subsequent restriction analysis as described previously [16].

Statistical analysis

Differences in variables between the groups were analysed using Kruskal-Wallis ANOVA, observed vs. expected frequencies tested by chi-square test. Univariate Cox regression analysis was used for detection of possible confounding factors. Cox proportional model adjusted for confounders was used to evaluate hazard ratios for NOS3 SNP. For all standard analysis Statistica for Windows (Statsoft Inc., Tulsa, OK, USA) was used. P ≤ 0.05 was considered statistically significant.

Results

At the end of the follow-up period, the cumulative incidence of DN progression reached 26.5 % (n = 272), in particular 4.2 % progressed from normo- to microalbuminuria, 1.4 %

Table 2. Results of univariate Cox regression analysis

Parameter	DN progression		Major cardiovascular event		All-cause mortality	
	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P
Age at enrolment	1.005 (0.984-1.026)	NS	1.028 (1.005-1.052)	0.017	1.045 (1.022-1.068)	<0.001
Sex (men)	0.724 (0.452-1.161)	NS	1.101 (0.668-1.813)	NS	0.917 (0.580-1.450)	NS
Duration of diabetes	1.025 (0.999-1.051)	NS	1.045 (1.018-1.072)	0.001	1.046 (1.021-1.072)	<0.001
BMI	0.993 (0.953-1.034)	NS	0.967 (0.919-1.017)	NS	0.962 (0.918-1.009)	NS
SBP	1.003 (0.988-1.017)	NS	0.999 (0.984-1.014)	NS	1.002 (0.988-1.017)	NS
DBP	0.988 (0.962-1.015)	NS	0.975 (0.947-1.003)	NS	0.971 (0.944-0.998)	0.034
GFR	0.148 (0.074-0.296)	<0.001	0.158 (0.070-0.354)	<0.001	0.136 (0.060-0.306)	<0.001
HbA _{1c}	0.977 (0.820-1.165)	NS	0.972 (0.818-1.154)	NS	0.919 (0.778-1.085)	NS

Abbreviations: BMI, body mass index; CI, confidence interval; DBP, diastolic blood pressure; DN, diabetic nephropathy; GFR, glomerular filtration rate; HbA_{1c}, glycated haemoglobin; HR, hazard ratio; SBP, systolic blood pressure

from normo- to macroalbuminuria, 31.9 % from micro- to macroalbuminuria, 15.3 % from microalbuminuria to ESRD and 47.2 % from macroalbuminuria to ESRD. Cardiovascular event was reached in 19.9 % of subjects from whom 48.4 % had non-fatal cardiovascular event and 61.3 % had fatal cardiovascular event. Of those with fatal cardiovascular event 15.8 % had a non-fatal cardiovascular event followed by fatal cardiovascular event during the follow-up period, for the purpose of the analysis however only the first, earlier one was considered. Cumulative incidence of all-cause mortality was 23.8 %.

Overall SNP genotyping success rate was 100 %. Genotype frequencies were 49.8 % GG, 40.3 % GT and 9.9 % TT, Hardy-Weinberg equilibrium was not violated ($P = \text{NS}$, chi-square test). In order to reveal possible non-genetic confounders we applied univariate Cox regression analysis (Table 2). GFR was identified as a confounder of DN progression ($P < 0.001$). Age at enrolment ($P = 0.017$), diabetes duration ($P = 0.001$) and GFR ($P < 0.001$) were found as confounders of major cardiovascular event and all-cause mortality ($P < 0.001$ for age; $P < 0.001$ for diabetes duration and $P < 0.001$ for GFR). Cox regression analysis of DN progression ($n = 272$) adjusted for age, diabetes duration and GFR demonstrated GT genotype as a predictor of DN progression (hazard ratio (HR) 1.843 [95 % CI 1.088 – 3.119], $P = 0.023$). The dominant model (i.e. genotypes containing minor allele were grouped together and compared to remaining homozygote) generated negative result ($P > 0.05$). Using the same approach, genotype TT was identified as a risk factor for major cardiovascular event (HR = 2.515 [95 % CI 1.060 – 5.965], $P = 0.036$) and again no difference was found in a dominant model ($P > 0.05$). Finally, no significant contribution of the NOS3 894G>T genotypes to all-cause mortality was ascertained ($P > 0.05$).

Discussion

In the present study, we identified genetic variant 894G>T in the NOS3 gene as a risk factor for the progression of DN in T2DM, therefore, we ascertained the role of NOS3 as a modifier gene for the clinical course of DN. Furthermore, NOS3 894G>T was identified as a risk factor for the major cardiovascular event in T2DM patients. Numerous SNPs in the NOS3 have been described, some of them associated with various diseases such as hypertension and coronary artery disease [17, 18] and – in case of 894G>T – with susceptibility to DN in our previous study [8]. A number of studies (of various sample size, ethnicity and inclusion criteria though) reported conflicting results regarding the relationship between NOS3 894G>T SNP and susceptibility to diabetic complications incl. DN. Several meta-analyses of case-control studies were performed so far on the NOS3 894G>T in DN. Chronologically,

the first and only one so far [19] that stratified for diabetes type have shown association of 894G>T with DN in Caucasians with T2DM. Other two meta-analyses focusing on NOS3 894G>T variant in DN in several populations identified 894G>T variant as a risk factor for DN in Asian but not Caucasian populations [20, 21]. Recent meta-analysis analysed association of the 894G>T variant with ESRD and found the T allele associated with ESRD susceptibility in overall population (including Caucasian, Asian, Brazil and African populations) and in Asians but not Caucasians separately [22].

Number of studies previously investigated functional impact of the NOS3 894G>T missense polymorphism. Experimental *in vitro* data indicated that amino acid change generates protein with higher susceptibility to cleavage with subsequent functional effect [23]. Furthermore, NO production was significantly lower in stably transfected CHO 894T cells [24]. Additionally, diabetic carriers of the 894T allele exhibited significantly lower serum NO levels [25]. However, other studies, on the contrary, did not find any functional effect of this SNP [26, 27]. We have to nevertheless bear in mind that NOS3 894G>T might be a mere marker of another linked functional variant, therefore, the proof of functionality is not automatically proof of causality. What is more important in the context of the current study is that functional consequences of the NOS3 genetic variability might not only affect the onset of DN but – due to the complex role of eNOS/NO axis in the regulation of renal haemodynamic – also modulate its clinical course and severity. The role of NOS3 as a modifier gene has only been studied in limited extent in renal pathology so far (only in the polycystic kidney disease [28, 29]). To our knowledge this is the first attempt to analyse NOS3 as a modifier gene in DN and we ascertained statistically significant effect of the 894G>T on the progression of DN.

The studied SNP was also significantly associated with the occurrence of the major cardiovascular event in T2DM subjects. Possible link between NOS3 894G>T and cardiovascular morbidity and mortality has been widely studied, unfortunately, most studies published so far were performed on non-Caucasian populations. Four recent meta-analyses found association of NOS3 SNP with ischemic stroke [30–32] or coronary heart disease [33] in Asian populations. No such association was found for Caucasian population. SNP 894G>T was also associated with susceptibility to cardiovascular disease normoalbuminuric type 1 diabetic subjects [34].

Conclusion

Using the prospective cohort of precisely clinically characterised T2DM subjects we demonstrated association of NOS3 variant 894G>T with DN progression and major cardiovascular event. NOS3 gene can be considered modifier gene for DN, however, replication studies and more comprehensive genetic characterisation are warranted.

Conflict of Interests

None declared.

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