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ORIGINAL ARTICLE

POLYMORPHISM OF ANGIOTENSIN-CONVERTING ENZYME (rs4340) AND DIABETIC NEPHROPATHY IN CAUCASIANS WITH TYPE 2 DIABETES MELLITUS

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ABSTRACT

Diabetic nephropathy (DN) is the leading cause of end-stage renal disease (ESRD) in developed countries. Several environmental and genetic factors predict the development and progression of DN. The renin-angiotensin system was demonstrated to be involved in the development of DN. We evaluated the association between rs4340 of the angiotensin-converting enzyme (*ACE*) gene and DN in Caucasians with type 2 diabetes mellitus (T2DM) in 276 Slovenian patients with T2DM who had DN, and 375 patients without clinical signs of DN. Genetic analysis was performed with either standard polymerase chain reaction (PCR) (for rs4340). Results were analyzed using the χ^2 test and multivariate logistic regression analyses. We found no association between rs4340 and DN. Cystatin C was significantly higher in the DN+ group (p <0.001) than in the DN group. Cystatin C was a better marker for the estimation of renal function than estimated glomerular filtration rate (eGFR) according to the modification diet in renal disease (MDRD) equation mL/min. We concluded that there was no association between the rs4340 of the *ACE* gene and DN in Caucasian patients who have T2DM.

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Keywords: Angiotensin-converting enzyme (ACE); Diabetic nephropathy; Insertion/deletion (I/D) gene polymorphism; Type 2 diabetes mellitus (T2DM).

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a multifactorial chronic metabolic disease characterized by post-prandial hyperglycemia that causes long-term macrovascular or microvascular complications. Microvascular complications are diabetic nephropathy (DN), neuropathy and diabetic retinopathy (DR) [1,2]. Diabetes mellitus (DM) is the most common cause of chronic kidney disease and end-stage renal disease [1,2]. In the pathogenesis of DN several environmental, genetic, and epigenetic factors are involved in complex interactions [3-5].

In DN, there is a major decrease in glomerular filtration rate (GFR) together with a rise in the excretion of proteins in urine [6]. The pathogenesis of DN is related to uncontrolled or chronic hyperglycemia and is characterized by hypertrophy of glomeruli, hyperperfusion, thickening of basement membranes and glomerular hyperfiltration. There is microalbuminuria and subsequently, progressive glomerulosclerosis, but tubulointerstitial fibrosis may occur, eventually leading to reduction in GFR [1,2].

In progression of DM and its complications, many risk factors are involved, *e.g.*, hypertension, dyslipidemia, smoking, obesity, aging and insulin resistance [7,8]. Clinically, non pharmacological interventions such as strict glycemic and blood pressure control, decrease in smoking and in dietary protein intake, have been shown to slow the progression of DN. The most validated clinical strategy

for slowing disease progression is therapeutic targeting of the renin-angiotensin aldesterone system (RAAS) [9,10].

Genetic polymorphisms of the RAAS system may affect the progression of DM and its complications, whereby angiotensinogen, angiotensin receptor and angiotensin-converting enzyme (*ACE*) gene polymorphisms have been implicated in the pathophysiology of DN [11]. Angiotensin-converting enzyme converts angiotensin I into active octapeptide angiotensin II, and inactivates bradykinin *via* the kalikrein-kininogen system [12].

The ACE insertion/deletion (I/D) gene polymorphism (rs4340) is a 287 bp sequence of DNA in intron 16 of the *ACE* gene on chromosome 17q23, whereas there are a few polymorphisms that are in a linkage disequilibrium (rs 4341, rs4646994) in some populations [13]. In adults, plasma ACE does not change with age and it is only influenced by environmental or lifestyle factors to a minor extent [15-17]. Compared with rs4340 insertion/insertion (I/I) homozygotes, circulating ACE levels in plasma were found to be nearly 30.0 and 60.0% higher in I/D heterozygotes and deletion/deletion (D/D), homozygotes, respectively [14]. A meta-analysis of studies on glomerulosclerosis reported that the overall frequency of the D allele was 54.0% [13]. Overall frequency of the D allele was unrelated to gender but there were ethnic differences [13].

The *ACE* I/D gene polymorphism, which correlates with circulating ACE concentration, may be implicated in the etiology of DN, but it has been poorly investigated while giving inconsistent results [18-20]. The present study was undertaken to evaluate the association between rs4340 of the *ACE* gene and DN in Caucasian patients with T2DM.

MATERIALS AND METHODS

We enrolled 651 unrelated Caucasians with T2DM from outpatient clinics of the University Medical Centre Maribor and the General Hospitals in Murska Sobota and Slovenj Gradec, Slovenia. Two hundred and seventy-six patients also had DN (cases) and 375 subjects with T2DM of more than 10 years duration but no clinical signs of DN (controls) were enrolled in the study. Diagnosis of DN was made according to the World Health Organization 1999 diagnostic criteria [16].

We excluded patients with overt nephropathy, poor glycemic control, significant heart failure New York Heart Association classification II-IV (NYHA II-IV), alcoholism, infection and other causes of renal disease. The study was approved by the National Medical Ethics Committee and was performed in compliance with the Helsinki declaration. After informed consent for participation in the study was obtained from all patients, a detailed interview was made.

Biochemical Analyses. Total serum cholesterol, low-density lipoproteins (LDL), high-density lipoproteins (HDL), triglycerides (TGs), serum cystatin C, fasting-serum glucose, serum glycemic Hb (Hb A_{1c}), serum urea, serum creatinine were determined by standard biochemical methods. Albumin-to-creatinine ratio was also determined for each patient in three urine samples, according to the diagnostic criteria. To assess the kidney function, we used the modification diet in renal disease (MDRD) equation and serum-cystatin C [21,22].

Genotyping. Genomic DNA was extracted from 100 µL of whole blood using a Qiagen isolation kit (Qiagen GmbH, Hilden, Germany) following the blood and body fluid spin »V3« protocol. The protocol was supported by five different reagents (QIAgene DNA Blood Mini Kit; Qiagen GmbH): buffer AL, 96.0% ethanol, buffer AW1, buffer AW2, buffer AE and appropriate amount of proteases (285 µL of proteases/200 µL of blood). From 200 µL of blood, 3-12 mg of genomic DNA, *i.e.*, 30-40 ng/mL were extracted according to the instructions of the manufacturer.

The protocol for rs4340 polymorphism of the *ACE* gene was as follows: one cycle at 94 °C for 5 min. followed by 30 cycles (94 °C for 60 seconds, 58 °C for 60 seconds and 72 °C for 60 seconds) and finishing with one cycle at 72 °C for 5 min.

Statistical Analyses. Statistical analyses were conducted with the use of the Statistical Package for the Social Sciences program for Windows, version 20 (SPSS Inc., Chicago, IL, USA). Continuous clinical data were compared by unpaired Student's *t*-test, while the χ^2 test was used to compare discrete variables. Data were expressed as mean ± SD (standard deviation) (continuous variables) or as the number and percent of patients (categorical variables). All variables that showed significant differences by univariate analysis (with a p values of <0.05 were considered significant) were analyzed together using a logistic regression method. A p value of <0.05 was considered statistically significant. Deviation from Hardy-Weinberg equilibrium (HWE) was assessed by the exact test (http://ihg. gsf.de/).

RESULTS

The demographic and clinical data of the case and control groups are shown in Table 1. There were no significant differences between the groups with respect to age, sex, duration of diabetes, diastolic blood pressure,

body mass index (BMI), smoking status, duration of DR, serum hemoglobin (Hb), estimated glomerular filtration rate (eGFR), TG and total HDL, LDL and cholesterol levels. However, statistically significant differences were observed in the duration of hypertension, systolic blood pressure, Hb A_{1c}, fasting-serum glucose, serum urea, serum creatinine, and urine albumin/creatinine ratio.

Differences in parameters reflecting renal function (serum creatinine, cystatin C, eGFR and urine albumin/creatinine ratio) confirmed chronic kidney disease in the DN+ group. Cystatin C was a better marker for the assessment of renal function than eGFR (MDRD equation mL/ min.). Cystatin C was significantly higher in the DN+ group (p <0.001) than in the control group. Univariate analysis demonstrated a statistically significant difference in genotype distribution in rs4340 genotypes (Table 2).

We used logistic regression analysis to evaluate whether this single nucleotide polymorphism (SNP) was independently associated with DN after adjusting for duration of hypertension, systolic blood pressure, cardiovascular disease, DR, diabetic foot, Hb A_{1c}, serum fasting glucose, serum urea, serum creatinine, serum cystatin C, urine albumin/creatinine ratio (g/mol), and found no statistically significant association of rs4340 with DN (Table 3).

Table 1. Clinical and laboratory characteristics of case and control groups

Parameters	**Cases (DN+)**	**Controls (DN–)**	***p* Value**
Number	276	375	
Gender (% males)	59.1	52.4	0.1
Age (years)	64.75 ± 9.15	63.75 ± 8.0	0.13
Duration of T2DM (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**
Systolic blood pressure (mmHg)	155.27 ± 18.92	149.84 ± 19.63	**<0.001**
Diastolic blood pressure (mmHg)	84.87 ± 11.63	84.06 ± 11.42	0.36
Body mass index	31.30 ± 4.68	30.77 ± 5.00	0.23
Active smokers (%)	6.6	8.9	0.31
Serum Hb A_{1c} (%)[a]	7.98 ± 1.38	7.65 ± 1.14	**0.001**
Serum-fasting glucose (mmol/L)	9.03 ± 2.76	8.51 ± 2.53	**0.01**
Hb (g/dL)	13.94 ± 1.49	13.94 ± 1.29	0.99
Serum urea (mmol/L)	7.35 ± 3.73	6.26 ± 1.91	**<0.001**
Serum creatinine (μmol/L): Males[b] Females[c]	93.13 ± 58.21 101.57 ± 61.84[b] 79.70 ± 49.21[c]	78.44 ± 20.15 84.28 ± 19.93[b] 71.91 ± 18.35[c]	**<0.001** **<0.001**[b] **<0.001**
eGFR (MDRD equation; mL/min.): Males[b] Females[c]	72.60 ± 19.74 71.97 ± 19.45[b] 74.31 ± 20.72[c]	75.22 ± 15.16 77.66 ± 14.33[b] 72.45 ± 15.69[c]	0.22 **0.002**[b] 0.13[c]
Serum cystatin C (mg/L)	0.95 ± 0.48	0.78 ± 0.21	**<0.001**
Serum total cholesterol (mmol/L)	4.62 ± 1.17	4.55 ± 0.99	0.42
Serum HDL cholesterol (mmol/L)	1.23 ± 0.35	1.26 ± 0.36	0.29
Serum LDL cholesterol (mmol/L)	2.59 ± 0.95	2.57 ± 0.80	0.73
Serum TG cholesterol (mmol/L)	2.08 ± 1.60	1.83 ± 1.24	0.04
Urine albumin/creatinine ratio (g/mol); sample #1	27.49 ± 55.46	1.57 ± 3.05	**<0.001**
Urine albumin/creatinine ratio (g/mol); sample #2	23.13 ± 39.34	1.60 ± 3.67	**<0.001**
Urine albumin/creatinine ratio (g/mol); sample #3	23.36 ± 42.49	1.62 ± 2.49	**<0.001**

DN+: cases; DN–: controls; T2DM: type 2 diabetes mellitus; Hb: hemoglobin; eGFR: estimated glomerular filtration rate; MDRD: modification diet in renal disease; HDL: high-density lipoproteins; LDL: low-density lipoproteins; TG: triglycerides. The values represent mean ± SD (standard deviation); statistically significant results are bold.

[a] The average value for Hb A_{1c}.
[b] Comparison of men who are DN+ *vs.* men who are DN–.
[c] Comparison women who are DN+ *vs.* women who are DN–.

Table 2. Distribution of the rs4340 genotypes and alleles in DN+ patients (cases) and in DN– controls.

Parameters	Cases (*n* = 276)	Controls (*n* = 375)	*p* Value
rs4340			
DD genotype (%)	90 (32.8)	115 (30.7)	
ID genotype (%)	143 (51.7)	169 (45.0)	0.02
II genotype (%)	43 (25.5)	91 (24.3)	
D allele (%)	323 (58.5)	399 (53.2)	0.06
I allele (%)	229 (41.5)	351 (46.8)	
PHWE[a]	0.3	0.07	

[a] PHWE: values were computed using Pearson's goodness-of-fit χ^2 (1 df) test.

Table 3. Association of rs4340 polymorphisms with DN in Caucasians with logistic regression.

Inheritance Model	Genotype	Cases (*n*=276)	Controls (*n*=375)	Unadjusted OR (95% CI)/*p* Value	Adjusted OR (95% CI)/*p* Value[a]
rs4340					
Codominant	DD	90 (32.8%)	115 (30.7%)	1.03 (0.39-2.72)/0.9	0.80 (0.29-2.25)/0.7
	ID	143 (51.7%)	169 (45.0%)	1.54 (0.61-3.90)/0.4	1.32 (0.49-3.53)/0.6
	II	43 (15.5%)	91 (24.3)	Reference	Reference

OR: odds ratio; 95% CI: 95% confidence interval.

[a] *p* Values were adjusted for duration of hypertension, systolic blood pressure, cardiovascular disease, DR, diabetic foot, Hb A_{1c}, serum-fasting glucose, serum urea, serum creatinine, serum cystatin C, urine albumin/creatinine ratio (g/mol): samples #1/#3.

DISCUSSION

In our cross-sectional study we found no association between rs4340 of the *ACE* gene and DN in Caucasians with T2DM. Conflicting reports have been made in different populations on the association between the I/D polymorphism in the *ACE* gene (rs4340) and DN. Our findings are in accordance with the study in French subjects with T2DM, but they differ from most studies performed in Chinese, Japanese and Indian populations, as well as in two meta-analyses in Asian and Arab patients with T2DM [13,19,20,23,25-29].

Wide inter-ethnic differences have been reported for allele/genotype frequencies of the I/D polymorphism of the *ACE* gene (rs4340), and these differences have been assumed to be responsible for the contradictory results among association studies in subjects with DN [13,19, 20,23-26]. An association between rs4340 and DN was demonstrated in Asian populations (Japan, South India) [20,23] but not in Iran [31] and Malaysia [32]. A meta-analysis has demonstrated that the *ACE* I/D gene polymorphism affected the development of DN, *i.e.*, that the D allele was a risk factor for DN, and I allele was a protective factor against DN in T2DM in an Asian population [26,33]. Another meta-analysis [27] reported that *ACE* I/D gene polymorphism (rs4340) was associated with T2DM in an Arab population. Moreover, the D/D genotype (rs4340) was reported to be an independent risk factor for renal end points in Chinese patients with T2DM [24]. Wang *et al.* [34] have recently demonstrated an association between the D allele (rs4340) and glomerular filtration impairment in Chinese patients with T2DM.

There were only a few reports in Caucasians [25,28, 29]. In a large case-control study, which enrolled patients in France, Finland and Denmark, it was shown that the rs4340 was associated with DN in subjects with type 1 diabetes mellitus (T1DM) [29]. Additionally, the D allele (rs4340) was reported to be an independent risk factor for both the onset and the progression of DN in T1DM patients [30]. Moreover, Yu *et al*. [25] reported that the DD genotype (rs4340) was associated with the end-stage renal disease in DN patients with T2DM. Contrary to the association between rs4340 and DN in T1DM, Hadjadj *et al.* [28] were not able to demonstrate an association between the rs4340 and DN in French subjects with T2DM, whereas so far, there have been no reports of rs4341 and DN in Caucasians.

A limitation of our study was the use of cross-sectional data in the analyses, restricting the possibility of causal inferences from our data. Another limitation of the study was the sample size. The strength of our cross-sectional study was a rather large community-based sample

of Caucasians with T2DM, and the detailed phenotypic characterization of the subjects with DN. In conclusion, rs4340 of the *ACE* gene was not demonstrated to be a potential genetic marker for DN in Caucasians with T2DM.

Declaration of Interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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