ORIGINAL ARTICLE

ASSOCIATION OF THE ACE rs4646994 AND rs4341 POLYMORPHISMS WITH THE PROGRESSION OF CAROTID ATHEROSCLEROSIS IN SLOVENIAN PATIENTS WITH TYPE 2 DIABETES MELLITUS

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ABSTRACT

The current study was designed to reveal possible associations between the angiotensin-converting-enzyme (*ACE*) gene polymorphisms (rs4646994 and rs4341) with markers of carotid atherosclerosis in patients with type 2 diabetes mellitus (T2DM) in a 4-year-long follow-up study. Five hundred and ninety-five T2DM subjects and 200 control subjects were enrolled. Genotyping of ACE polymorphisms was performed using KASPar assays, and ultrasound examinations were performed twice (at the enrollment and at follow-up). With regard to the progression of atherosclerosis in subjects with T2DM, statistically significant differences were demonstrated in the change of the sum of carotid plaques thickness for the rs4646994 polymorphism. We did not demonstrate an association between the tested polymorphisms (rs4646994 and rs4341) and either carotid intima media thickness (CIMT) or CIMT progression in a 3.8-year period. In our study, we demonstrated that subjects with T2DM with the DD genotype of the rs4646994 [ACE insertion/deletion (I/D)] polymorphism had faster progression of atherosclerosis in comparison to subjects with other genotypes.

Keywords: Angiotensin-converting-enzyme (*ACE*) gene polymorphism; Association study; Carotid atherosclerosis; Type 2 diabetes mellitus (T2DM).

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INTRODUCTION

Type 2 diabetes mellitus (T2DM) represents a chronic illness characterized by the disability of the body to utilize glucose either because of insulin resistance in peripheral tissues or because of a decreased production of insulin by the pancreas [1]. Type 2 diabetes mellitus is known to promote the atherosclerotic process, which is characterized by endothelial dysfunction and by accumulation of foam cells and vessel wall inflammation. As the process continues, the narrowing of the vessel lumen occurs, leading to acute cardiovascular events [2].

The renin-angiotensin-aldosterone system is one of the main regulators of blood pressure having also other local (tissue-specific) roles [3]. Genetic polymorphisms in different parts of this system have previously been described to associate with various cardiovascular and other diseases, with the angio-

tensin-converting-enzyme (ACE) insertion/deletion (I/D) polymorphism representing one of the most commonly studied polymorphisms that affects circulating ACE levels [3]. This polymorphism has recently been shown to be in linkage disequilibrium with another ACE polymorphism, rs4341 [3]; however, data about these two polymorphism and their possible association with carotid atherosclerosis in patients with diabetes mellitus are limited.

The present study was thus designed to investigate the association between polymorphisms of the *ACE* gene (rs 4646994 and rs4341) and markers of carotid atherosclerosis [carotid intima media thickness (CIMT), number of affected segments of carotid arteries and sum of plaques thickness] in patients with T2DM. The second aim was to see whether these two polymorphisms (rs4646994 and rs4341) affect progression of carotid atherosclerosis in a 4-year follow-up.

MATERIALS AND METHODS

In this cross-sectional study, 595 (338 males; 257 females) subjects with T2DM and 200 (92 males; 108 females) subjects without T2DM (control group) were enrolled as described previously [4]. The study protocol was approved by the Slovene Medical Ethics Committee (128/09/ 2010). After informed consent for participation in the study was obtained, a detailed interview was made.

All ultrasound examinations were performed by two experienced doctors blinded to the participants' diabetes status. The CIMT, defined as the distance from the leading edge of the lumen-intima interface to the leading edge of the media-adventitia interface, was measured as described previously [4]. Plaques were defined as a focal intima-media thickening and divided according to their echogenic/ echolucent characteristics into five types as described previously [4]. The inter-observer reliability for carotid plaque characterization was found to be substantial (κ = 0.64, p <0.001).

Blood samples for biochemical analyses were collected as described previously [4]. The genomic DNA was extracted from 100 µL of whole blood using a FlexiGene DNA isolation kit, in accordance with the recommended protocol (Qiagen GmbH, Hilden, Germany). The ACE polymorphisms (rs4646994 and rs4341) were determined by a novel fluorescence-based competitive allele-specific polymerase chain reaction (PCR) (KASPar; Kbioscience Ltd., Hoddesdon, Hertfordshire, UK), assay. Details of the method used can be found at http://www.kbioscience. co.uk/.

Continuous variables were expressed as means ± standard deviations (SDs) if normally distributed, and as median (interquartile range) if asymmetrically distributed. Continuous clinical data were compared using an unpaired Student's *t*-test or analysis of variance (ANOVA) when normally distributed and the Mann-Whitney U test or the Kruskal-Wallis H test when asymmetrically distributed. The Pearson χ^2 test was used to compare discrete variables.

To determine the association of the *ACE* gene polymorphisms (rs4646994 and rs4341) with CIMT, a multiple linear regression analysis was performed. We used an additive model in which common allele homozygotes were coded as 1, heterozygotes as 2, and rare allele homozygotes as 3. All the regression models were adjusted for the presence of well-established cardiovascular risk factors. The results are presented as standardized β coefficients and *p* values for the linear regression and by odds ratios (ORs) and 95% confidence intervals (CIs) for the logistic regression. A two-tailed *p* value of less than 0.05 was considered statistically significant. A statistical analysis was performed using the Statistical Package for the Social Science (SPSS) software for Windows, version 20 (SPSS Inc., Chicago, IL, USA).

RESULTS

Patients with T2DM had a greater waist circumference, higher fasting glucose and Hb A_{1c} levels compared to controls, whereas there were no statistically significant differences in age, body mass index (BMI), and systolic and diastolic blood pressure between patients with T2DM and control subjects (Table 1). Patients with T2DM had lower total, high density lipoprotein (HDL) and low density lipoprotein (LDL) cholesterol levels and higher triglyceride levels compared to controls (Table 1). Plasma levels of inflammatory markers (*i.e.,* high sensitive C-reactive protein (hs-CRP) were statistically significantly higher in patients with T2DM compared to controls (Table 1).

The genotype distributions both in patients with T2DM and controls were in the Hardy-Weinberg equilibrium for both *ACE* gene polymorphisms

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Table 1. Baseline characteristics of subjects with type 2 diabetes mellitus and control subjects without type 2 diabetes mellitus.

Parameters	T2DM Subjects (n = 595)	Control Subjects (n = 200)	p Value
Age (years)	61.38 ± 9.65	60.07 ± 9.18	0.07
Gender: males females	338 (56.8%) 257 (43.2%)	92 (46.0%) 108 (54.0%)	**0.008**
Duration of T2DM (years)	11.25 ± 7.88	–	–
Cigarette smokers (%)	53 (8.9%)	34 (17.0%)	**0.002**
Waist circumference (cm)	108.65 ± 12.88	93.31 ± 13.18	**<0.001**
BMI (kg/m^2)	30.96 ± 4.74	27.90 ± 4.42	0.16
SBP (mmHg)	146.98 ± 19.98	143.30 ± 16.6	0.86
DBP (mmHg)	85.75 ± 11.62	84.70 ± 11.6	0.19
Fasting glucose (mmol/L)	8.04 ± 2.57	5.27 ± 0.87	**<0.001**
Hb A$_{1c}$ (%)	7.89 ± 3.56	4.79 ± 0.29	**<0.001**
Total cholesterol (mmol/L)	4.70 ± 1.19	5.36 ± 1.08	**<0.001**
HDL cholesterol (mmol/L)	1.19 ± 0.35	1.43 ± 0.37	**<0.001**
LDL cholesterol (mmol/L)	2.63 ± 0.94	3.24 ± 0.98	**<0.001**
Triglycerides (mmol/L)	1.9 (1.2-2.7)	1.3 (0.9-1.9)	**<0.001**
hs-CRP (mg/L)	2.2 (1.0-4.3)	1.3 (0.8-2.7)	**<0.001**
CIMT (μm)	1013.0 ± 208.0	979.0 ± 141.0	0.03

Continuous variables are expressed as means ± SDs if normally distributed and as median (interquartile range) if asymmetrically distributed. Categorical variables are expressed as frequency (percentage). BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; Hb A$_{1c}$: glycated hemoglobin (Hb); HDL: high density lipoprotein; LDL: low density lipoprotein; hs-CRP: high sensitivity C-reactive protein; CIMT: carotid intima media thickness.

[rs4646994: T2DM (genotype frequencies: II genotype 21.5%, ID genotype 46.4%, DD genotype 32.1%; χ^2 = 2.27; p = 0.13) and controls (genotype frequencies: II genotype 24.0%, ID genotype 49.0%, DD genotype 27.0%; χ^2 = 0.07; p = 0.78); rs4341: T2DM (genotype frequencies: GG genotype 28.1%, GC genotype 52.1%, CC genotype 19.8%; χ^2 = 1.44; p = 0.23) and controls (genotype frequencies: GG genotype 23.5%, GC genotype 54.5%, CC genotype 22.0%; χ^2 = 1.63; p = 0.20)]. No statistically significant differences in the ACE rs4646994 and rs4341 genotype distribution frequencies were observed between the T2DM patients and controls. Moreover, in our study, linkage disequilibrium between the two selected single nucleotide polymorphisms (SNPs) (rs4646994 and rs4341) was confirmed (d' =0.98 r^2=0.82).

Several parameters of carotid atherosclerosis, such as CIMT, number of involved segments, and sum of plaque thicknesses, were evaluated with regard to different genotypes of both ACE polymorphisms in subjects with T2DM at enrollment and after 3.8 years (Table 2). Moreover, the parameters of progression of atherosclerosis, *i.e.,* annual increase in CIMT, change in number of segments with plaques

and change in the sum of carotid plaque thicknesses, were analyzed with univariate and multiple linear regression analyses (Tables 3 and 4). With regard to the progression of atherosclerosis in subjects with T2DM, statistically significant differences were demonstrated in the change of the sum of carotid plaque thickness for the rs4646994 polymorphism only (Table 3). Finally, according to the results of multiple linear regression analysis, faster progression of atherosclerosis was demonstrated in subjects with T2DM with the DD genotype of the rs 4646994 (ACE I/D) polymorphism in comparison with subjects with other genotypes (Table 4).

DISCUSSION

In this study, we demonstrated the effect of the DD genotype of the rs4646994 (ACE I/D) polymorphism on atherosclerosis progression in subjects with T2DM. Statistically significant differences in a 3.8-year observation period were found in the change in the sum of carotid plaques for the rs4646994 polymorphism. Our findings are in accordance with the findings of Saitou *et al.* [5], who on a cohort of 222

Table 2. Comparison of markers of carotid atherosclerosis in subjects with type 2 diabetes mellitus at the beginning and the end of the study with regard to the rs4646994 (angiotensin-converting-enzyme insertion/deletion) and rs4341 polymorphisms.

rs4646994 (ACE I/D)	Enrollment				Endpoint			
	II	ID	DD	*p* Value	II	ID	DD	*p* Value
Intima media thickness (μm)	998.0±147.0	1002.0±178.0	1012.0±178.0	0.62	1048.0±147.0	1054.0±128.0	1060.0±164.0	0.59
Involved segments (*n*)	2.65±1.49	3.04±1.71	2.66±1.44	0.12	4.48±1.75	4.62±1.68	4.71±1.76	0.68
Sum of plaque thickness (mm)	7.92±3.47	8.99±3.38	7.67±3.14	0.09	10.17±4.29	10.08±5.26	11.38±5.32	0.76
rs4341	Enrollment				Endpoint			
	GG	GC	CC	*p* Value	GG	GC	CC	*p* Value
Intima media thickness (μm)	1022.0±212.0	1002.0±217.0	1018.0±191.0	0.71	1065.0±155.0	1054.0±163.0	1051.0±164.0	0.62
Involved segments (*n*)	2.49±1.48	2.54±1.73	2.44±1.57	0.89	3.60±1.76	3.70±1.64	3.70±1.78	0.96
Sum of plaque thickness (mm)	7.77±4.50	8.02±4.42	7.53±4.77	0.74	9.38±5.34	9.16±5.16	8.72±4.56	0.88

ACE I/D: angiotensin-converting-enzyme insertion/deletion.

Table 3. Changes of markers of carotid atherosclerosis in subjects with type 2 diabetes mellitus between first the examination and the examination at the end of the study with regard to the rs4646994 (angiotensin-converting-enzyme insertion/deletion) and rs4341 polymorphisms.

rs4646994 ACE I/D	II	ID	DD	*p* Value
Annual increase in CIMT (μm/year)	14.28 (5.35-26.83)	22.43 (16.73-32.19)	20.34 (10.53-33.65)	0.34
Δ Number of segments with plaques	3.0 (1.0-3.0)	1.0 (0.5-2.5)	2.0 (1.0-3.0)	0.42
Δ Sum of carotid plaques thickness (mm)	4.00 (2.30-5.30)	4.68 (3.30-7.60)	6.22 (3.90-8.10)	0.04
rs4341	GG	GC	CC	*p* Value
Annual increase in CIMT (μm/year)	21.05 (14.28-33.65)	20.69 (16.55-24.26)	14.28 (10.71-20.08)	0.26
Δ Number of segments with plaques	2.0 (1.0-3.5)	2.0 (1.0-2.5)	3.0 (2.0-3.0)	0.49
Δ Sum of carotid plaques thickness (mm)	4.60 (3.40-7.90)	5.60 (4.35-8.55)	5.6 (2.60-6.90)	0.07

ACE I/D: angiotensin-converting-enzyme insertion/deletion. Annual increase in CIMT (carotid intima media thickness) was calculated as CIMT(beginning)-CIMT(endpoint)/follow-up in years. Change in number of plaques is expressed as number of segments with plaque at the endpoint minus the number at the beginning. Sum of plaque thickness is calculated as the end sum minus the beginning sum. Data are expressed as median and range.

Table 4. Multiple linear regression analysis for association of rs4646994 (angiotensin-converting-enzyme insertion/deletion) with carotid atherosclerosis progression in patients with type 2 diabetes mellitus.

Parameters	Δ CIMT/Year		Δ Number of Segments		Δ Sum of Plaque Thickness	
	β	*p* Value	β	*p* Value	β	*p* Value
A) rs4646994						
Hypertension (yes/no)	0.144	0.59	0.206	0.88	0.272	0.53
Systolic blood pressure	0.031	0.42	0.027	0.15	0.014	0.69
ID	0.141	0.49	0.116	0.51	0.845	0.26
DD	0.102	0.63	0.146	0.41	0.952	**0.04**

All models were adjusted to age, sex, smoking habits, serum levels of Hb A_{1c}, statin treatment and initial values of the dependent variables. Reference groups are homozygotes for the I allele.

T2DM patients reported faster progression of atherosclerosis in subjects with the D allele.

Moreover, in our study, we did not demonstrate an association between tested polymorphisms (rs4646994, rs4341) and either CIMT or CIMT progression in an almost 4-year period. In 1994, it was reported for the first time that ACE levels correlate with CIMT in healthy persons [6]. As the ACE I/D polymorphism is known to affect circulating ACE levels (subjects with the DD genotype having the highest serum ACE levels compared to subjects with other genotypes) [7], subsequent studies have already been conducted to identify a possible association of the ACE I/D genotype with atherosclerosis, both on general and diabetic populations, providing contradictory results [5-10]. Using smaller or larger cohorts of the general population, association of the ACE I/D polymorphism with carotid atherosclerosis was confirmed in some studies [6-8] and opposed by others [9]. Finally, large meta-analyses confirmed statistically significant association of ACE I/D with CIMT in the general population [10].

In studies that did not focus on the general population but on patients with T2DM, Kogawa *et al.* [11] in 1997, studied femoral and CIMT in healthy persons and patients with T2DM, and showed that *only* in T2DM patients was there a significant association of the D allele with higher CIMT. Since the study of Kogawa *et al.* [11], studies focusing on the association of CIMT and the ACE I/D polymorphism provided contradictory results, similar to studies conducted in the general population. In the diabetic heart study no association was found between ACE I/D and CIMT [12], similar to the study focusing on the offspring of patients with T2DM [13]. On the other hand, Sticchi *et al.* [14] reported a higher risk of carotid stenosis in D allele carriers, and Zhou *et al.* [15] reported higher lipid levels in older patients with T2DM carrying the D allele, which could promote atherosclerosis progression.

Certain limitations of our study should be noted. First is the moderate sample size of our study. However, all the participants were enrolled from an ethnically homogenous population, which minimizes possible biases from population stratification. Second, the results of our study may be affected by statin therapy, and antihypertensive agents, and these facts were not appreciated in the statistical analysis.

In conclusion, our study represents the first larger study focusing on the effect of two ACE polymorphisms (rs4646994 and rs4341) on the progression of carotid atherosclerosis in subjects with T2DM. We demonstrated that those subjects with T2DM with the DD genotype of the rs4646994 (ACE I/D) polymorphism had faster progression of atherosclerosis in comparison with subjects with other genotypes.

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