Angiotensinogen (*AGT*) gene missense polymorphisms (rs699 and rs4762) and diabetic nephropathy in Caucasians with type 2 diabetes mellitus

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ABSTRACT

Gene polymorphisms associated with the renin–angiotensin–aldosterone system (RAAS) have been extensively studied in diabetic nephropathy (DN) patients, due to therapeutic potential of targeting the RAAS and slowing down the disease progression. The aim of our study was to examine the association between angiotensinogen (*AGT*) gene polymorphisms (rs699 and rs4762) and DN in Caucasians with type 2 diabetes mellitus (T2DM). A total of 651 unrelated Slovenian (Caucasian) T2DM patients were tested for *AGT* rs699 and rs4762 polymorphisms using a novel fluorescence-based kompetitive allele-specific polymerase chain reaction (KASPar) assay. A study group consisted of 276 T2DM patients with DN, while control group included 375 patients without DN but who have had T2DM for >10 years. For rs699 polymorphism, the frequencies of GG, GA and AA genotypes were 20.6%, 52.2% and 27.2%, respectively in T2DM patients and 23.4%, 48.1% and 28.5%, respectively in controls. The distributions of GG, GA and AA genotypes for rs4762 polymorphism were 73.9%, 23.2% and 2.9%, respectively in T2DM patients and 70.4%, 27.5% and 2.1%, respectively in controls. No significant differences in the allele frequencies were found between T2DM patients and controls for both polymorphisms. *AGT* rs699 and rs4762 missense polymorphisms are not associated with DN in our subset of Slovenian T2DM patients.

KEY WORDS: Angiotensinogen; AGT; rs699; rs4762; diabetic nephropathy; T2DM; type 2 diabetes mellitus
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INTRODUCTION

Diabetic nephropathy (DN) is a chronic, progressive microvascular complication of diabetes mellitus. It is associated with high cardiovascular morbidity and mortality and is still the most common cause of end-stage renal disease (ESRD) in developed countries [1-4]. DN is the result of interaction between environmental and genetic factors, where the letter are relatively common and probably linked genes, and these factors also include related epigenetic mechanisms [1,5,6]. Clinically, non-pharmacological interventions such as strict glycemic and blood pressure control, non-smoking habits, and dietary protein intake have been shown to slow the progression of DN. Nevertheless, the most common clinical strategy for slowing the disease progression is therapeutic targeting of the renin–angiotensin–aldosterone system (RAAS) [2-4,7,8].This is related to the pathophysiological relationship between DN and increased blood pressure, i.e. type 2 diabetes mellitus (T2DM) and DN are usually associated with hypertension [2,3,7]. The complex underlying mechanisms are still not completely understood and include excess sodium retention, sympathetic nervous system activation, endothelial cell dysfunction, increased oxidative stress, and RAAS activation [7]. All components of the systemic RAAS are also present in local (tissue) RAAS, and for example in the kidneys, the local effects accelerate the progression of renal disease [7,9]. Data suggest that the activation of intrarenal angiotensinogen (AGT) production plays an important role in the development of DN [10]. Urinary AGT was proposed as a possible early marker of DN, as it reflects intrarenal RAAS status in chronic glomerulonephritis [11].

Although RAAS gene polymorphisms have been extensively studied in diabetic and cardiovascular diseases, including several meta-analyses, contradictory results were reported

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[9,12-15]. *AGT* gene is located on the long arm of chromosome 1 (1q42-43), consisting of five exons and four introns [16]. *AGT* codes for 485 amino acids (AA), including: A signal peptide (AA 1-33), AGT chain (AA 34-485) and several other peptides, such as angiotensin I-III, which are all part of the RAAS. In brief, AGT is a plasma globulin belonging to the serpin family, synthetized in the liver. It is cleaved by renin which produces inactive decapeptide angiotensin I. Angiotensin-converting enzyme (ACE) converts angiotensin I to active octapeptide angiotensin II which increases aldosterone secretion, elevates blood pressure, and inhibits renin secretion by binding to angiotensin II receptors. ACE II forms angiotensin (1-7) from angiotensin II which has vasodilatory, antiproliferative, and apoptotic functions [17].

In this study, we investigated the possible effects of two missense single nucleotide polymorphisms (SNPs) of the *AGT* gene on DN. SNP rs699 is a T to C substitution in the exon 2, resulting in a functional methionine (M) to threonine (T) exchange at codon 268 (M268T). Previously, rs699 was positioned to the amino acid 235 and the SNP is therefore also referred to as M235T. The rs699 threonine variant is associated with higher plasma AGT levels and higher blood pressure [18,19].

SNP rs4762 is a C to T substitution in *AGT* exon 2 with a consequent functional threonine (T) to methionine (M) exchange at codon 207 (termed T207M or T174M). This polymorphism was not associated with higher plasma AGT levels [18].

The aim of our study was to examine the association between the *AGT* rs699 and rs4762 polymorphisms and DN in Caucasians with T2DM.

MATERIALS AND METHODS

Patients

A total of 651 consecutive, unrelated Slovenian (Caucasian) T2DM patients from outpatient clinics of the University Medical Centre Maribor, General Hospitals Murska Sobota and Slovenj Gradec, Slovenia, were enrolled in the study in the period from 2010 to 2014. The T2DM patients were classified into two groups according to the presence of DN. A study group consisted of 276 T2DM patients with DN (DN+, cases), while control group included 375 patients without DN but who have had T2DM for >10 years (DN–, controls). Due to the cross-sectional study design, the cases and controls were not matched for their demographic, lifestyle, or medical characteristics.

The diagnosis of T2DM and DN was made according to the World Health Organization diagnostic criteria [20]. Patients with overt nephropathy, poor glycemic control, significant heart failure (New York Heart Association [NYHA] II-IV), alcoholism, infection, and other causes of renal disease were excluded.

The study was approved by the national medical Ethics Committee and was performed in compliance with the Helsinki declaration. All patients were interviewed in person for lifestyle and medical information after signing an informed consent to participate in the study. The questions concerned age, sex, blood pressure, duration of T2DM and hypertension, body mass index (BMI), smoking status, incidence of microvascular complications of T2DM (diabetic retinopathy [DR], DN, diabetic foot [DF]), duration of DR, estimated glomerular filtration rate (eGFR), hemoglobin (Hb), total cholesterol, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol levels.

Biochemical analyses

Plasma glucose, Hb, glycated hemoglobin (HbA1c), urea, creatinine, cystatin C, total cholesterol, LDLs, HDLs, and triglycerides (TGs) were determined by standard biochemical methods. For each patient, albumin/creatinine ratio (ACR) was also determined in three urine samples according to diagnostic criteria.

Genotyping

Genomic DNA was extracted from 100 µl of peripheral blood using a Qiagen isolation kit (DNeasy Blood and Tissue Kit, Qiagen, Germany). The rs699 and rs4762 polymorphisms of the *AGT* gene were genotyped by LGC – Genomics Laboratories (Registered Office: LGC, Queens Road, Teddington, Middlesex, TW11 OLY, UK) using their novel fluorescence-based kompetitive allele-specific polymerase chain reaction [PCR] (KASPar) assay. The details of this method can be found at http://www.kbioscience.co.uk/. The following primers (5'-3') were used for PCR reaction:

- *AGT* (rs699)/F: CAGGGTGCT GTCCACACTGG ACCCC
- *AGT* (rs4762)/R:CCGTTT GTGCAGGGCCTGG CTCTCT.

Statistical analysis

Statistical analyses were conducted using SPSS program for Windows version 20 (SPSS Inc., Illinois, USA). The distribution of data was analyzed by the Kolmogorov–Smirnov test. Continuous variables were expressed as mean ± standard deviation (SD) when normally distributed, and compared by unpaired Student's *t*-test. The variables were expressed as median values (interquartile range [IQR]) when asymmetrically distributed, and compared using the Mann–Whitney *U* test. Chi-square test was used to compare discrete variables. Categorical variables were expressed as the number and

percentage of patients. Furthermore, all variables that showed significant differences by the univariate analysis were analyzed together in a logistic regression analysis (for both analyses $p < 0.05$ was considered statistically significant). The deviation from Hardy-Weinberg equilibrium (HWE) was assessed by the exact test (http://ihg.gsf.de/) [21].

RESULTS

The population characteristics are presented in Table 1. There were no significant differences between DN+ and DN– groups with respect to the age, sex, duration of T2DM, diastolic blood pressure (DBP), BMI, smoking status, duration of DR, eGFR, Hb, total cholesterol, HDL, and LDL cholesterol levels. However, there was a statistically significant difference in the duration of hypertension, systolic blood pressure (SBP), family history of cardiovascular disease (CVD), urine ACR, HbA1c, TG, fasting glucose, urea, and creatinine levels. Moreover, in DN+ group, a higher percentage of other chronic diabetic complications (i.e., DR and DF) was found compared to the patients without DN (Table 1).

Differences in parameters of kidney function (i.e., serum urea, creatinine, cystatin C, and urine ACR) were consistent with chronic kidney disease in DN+ group. These patients showed significantly more other chronic diabetic complications, such as DR and DF, and were more burdened with CVD, although no differences were found regarding their family history of CVD, smoking status, lipid profile, or diabetes duration. Importantly, they had worse glycemic control, higher SBP, and longer history of hypertension compared to the patients without DN (Table 1).

The genotype distribution and allele frequencies of *AGT* rs699 and rs4762 polymorphisms in DN+ and DN– groups are presented in Table 2. No significant differences in the allele frequencies were found between the two groups. In both groups, rs699 and rs4762 SNPs conformed to the HWE (DN+ group: rs699, $p = 0.43$; rs4762, $p = 0.28$; DN– group: rs699, $p = 0.47$; rs4762, $p = 0.58$).

Furthermore, no association between the two SNPs and DN was found in the multivariate logistic regression analysis adjusted for different confounders (i.e., duration of hypertension, SBP, CVD, DR, DF, HbA1c, fasting glucose, urea, creatinine, cystatin C, urine ACR), according to the co-dominant genetic model (Table 3).

DISCUSSION

In our study DN+ patients showed higher CVD morbidity compared to the controls, despite adjusted CVD family burden; furthermore, they showed higher BMI, and differences with regard to the smoking habits and lipid profile were also observed between the two groups. Hypertension contributes to the increased incidence of CVD in patients with DN [7]; therefore, blood pressure control is of special interest in these patients. There is also a relationship between T2DM, DN, and increased blood pressure. The latter is commonly present in patients with T2DM before DN development and leads to further progression of kidney disease in existing DN. Taking into account the beneficial effects of RAAS therapeutic targeting [2-4,7,8], the effect of RAAS gene polymorphisms on the development and progression of DN has been in focus. The RAAS genes have been studied extensively in both hypertension and control patients, with several well-designed meta-analyses that both confirmed and opposed the role of the RAAS gene polymorphisms in this condition [21].

Numerous studies also investigated the role of *AGT* rs699 polymorphism (M235T, M268T) in DM and chronic diabetic complications. Although an association with insulin resistance [22], serum AGT and blood pressure was noted [18,19], a systematic review of 73 studies by Rahimi et al. [9] indicated that this polymorphism does not affect the risk of DM and could not be associated with DR nor diabetic neuropathy [9]. In certain populations, the association between increased risk of DN and the polymorphic allele T or genotype TT was demonstrated (i.e., in two Indian populations [23,24], a Pakistani [25], Tunisian [26], Taiwanese [27], Japanese [28], Chinese [29], and Turkish population [30]). However, the reviews and extensive meta-analyses could not confirm the association of rs699 with DN [13,14,31,32]. Our results match these findings as we found no association between rs699 and DN in the T2DM population, and they also resemble the results obtained for Polish [33] and German population [34].

In contrast to rs699, *AGT* rs4762 polymorphic variant (T207M, T174M) was not included in the meta-analyses, as most individual studies did not show the association of rs4762 with DN [25,27] nor with ESRD [31]. The association of rs4762 with DN was only confirmed in Taiwanese population [27]. In addition, rs4762 was not associated with plasma AGT levels [18]. Similarly, we found no association between rs4762 and DN in our population.

Previous studies suggested that the association of *AGT* polymorphisms with different phenotypes varies across racial/ethnic groups [22,35], and this could potentially explain the negative results obtained in our study. Nevertheless, a meta-regression analysis was performed to evaluate the effect of ethnic heterogeneity on the results of association studies for various genes. They concluded that the lack of association of several genes (but not the *AGT*) with DN could be explained by ethnic heterogeneity [13]. Our results could also be related to the fact that DN is not the only cause of kidney disease in DM patients. Moreover, although renal biopsy is the gold standard for the diagnosis of DN, it is not commonly performed in DM patients. Instead, the diagnosis

TABLE 1. Population characteristics of T2DM patients with DN (DN+, cases) and patients without DN but who have had T2DM for >10 years (DN–, controls)

Characteristics	Cases (DN+)	Controls (DN–)	Significant (*p*)
Number	276	375	
Sex (M)	59.1%	52.4%	0.1
Age (years)	64.75±9.15	63.75±8.0	0.13
Duration of T2DM (years)	14.0 (10.0-19.0)	13.5 (11.0-18.3)	0.84
Duration of hypertension (years)	10 (5-17)	10 (4-15)	0.06
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
DN (%)	9.1	6.0	0.38
DF (%)	15.5	8.1	0.03
Serum-HbA1c (%)	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
Serum-Hb (g/L)	139.39±14.91	139.40±12.96	0.99
Serum-urea (mmol/L)	7.35±3.73	6.25±1.91	<0.001
Serum-creatinine (μmol/L)	81.0 (66.0-103.0)	76.0 (64.0-89.8)	0.002
Male sex	92.0 (71.5-107.0)*	82.5 (69.0-95.0)*	0.006
Female sex	70.5 (55.8-88.3)**	70.0 (59.0-81.0)**	0.7
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**
Serum-cystatin C (mg/L)	0.8 (0.7-1.1)	0.7 (0.6-0.9)	<0.001
Serum-total cholesterol (mmol/L)	4.62±1.17	4.55±0.99	0.42
Serum-HDL (mmol/L)	1.23±0.35	1.26±0.36	0.29
Serum-LDL (mmol/L)	2.59±0.95	2.57±0.80	0.73
Serum-TGs (mmol/L)	1.6 (1.1-2.5)	1.5 (1.0-2.3)	0.04
U-albumin/creatinine ratio (g/mol) - Sample Number 1	9.4 (4.5-33.6)	1.0 (0.6-1.6)	<0.001
U-albumin/creatinine ratio (g/mol) - Sample Number 2	10.6 (4.5-33.9)	1.0 (0.7-1.7)	<0.001
U-albumin/creatinine ratio (g/mol) – Sample Number 3	9.5 (4.3-33.9)	1.1 (0.7-1.8)	<0.001

*Comparing men with DN versus men without DN. **Comparing women with DN versus women without DN. Data are presented as mean±SD, proportions/percentages and median (interquartile range [IQR]) values. SBP: Systolic blood pressure; DBP: Diastolic blood pressure; BMI: Body mass index; CVD: Cardiovascular disease; DR: Diabetic retinopathy; DN: Diabetic neuropathy; DF: Diabetic foot; HbA1c: Glycated hemoglobin; Hb: Hemoglobin; eGFR: Estimated glomerular filtration rate; MDRD: Modification of Diet in Renal Disease; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; TGs: Triglycerides; U: Urine

TABLE 2. Distribution of *AGT* rs699 and rs4762 genotypes and alleles in patients with diabetic nephropathy (cases) and in those without diabetic nephropathy (controls)

Genotypes and alleles	Cases (276)	Controls (375)	*p* value
rs699			
GG	57 (20.6)	88 (23.4)	
GA	144 (52.2)	180 (48.1)	0.56
AA	75 (27.2)	107 (28.5)	
G allele (%)	258	356	0.8
A allele (%)	294	394	
PHWE†	0.43	0.47	
rs4762			
GG	204 (73.9)	264 (70.4)	
GA	64 (23.2)	103 (27.5)	0.41
AA	8 (2.9)	8 (2.1)	
G allele (%)	472	631	0.5
A allele (%)	80	119	
PHWE†	0.28	0.58	

†P_{HWE} values were computed using Pearson's goodness-of-fit Chi-square (1 degrees of freedom [df]). HWE: Hardy-Weinberg equilibrium

of DN is based on a combination of kidney disease and other specific characteristics [7,20]. Finally, our results are most likely the consequence of DN being a polygenic trait. Similarly, the results of other studies that identified genes or genome regions associated with DN were quite inconsistent [5]. Thus, the focus should not only be on single polymorphisms when investigating genetic basis of DN. In addition, epigenetic mechanisms are also involved in DN development (e.g., DNA methylation, chromatin histone modifications, and functional noncoding RNAs [6]), and therefore should be considered in future studies.

CONCLUSION

AGT rs699 and rs4762 missense polymorphisms are not associated with DN in our subset of Slovenian T2DM patients. Overall, our results contribute, at least partially, to better understanding of the genetic background of DN.

TABLE 3. Association of *AGT* rs699 and rs4762 polymorphisms with DN in the Slovenian (Caucasian) T2DM population according to logistic regression analysis

Inheritance model	Genotype	Cases (276)	Controls (375)	Unadjusted OR, 95% CI/*p* value	Adjusted OR, 95% CI/*p* value†
rs699					
Co-dominant	GG	57 (20.6)	88 (23.4)	0.92 (0.59-1.44)/0.72	1.12 (0.42-2.95)/0.82
	GA	144 (52.2)	180 (48.1)	1.14 (0.78-1.65)/0.50	1.65 (0.74-3.66)/0.22
	AA	75 (27.2)	107 (28.5)	Reference	Reference
rs4762					
Co-dominant	GG	204 (73.9)	264 (70.4)	0.80 (0.56-1.15)/0.64	0.94 (0.45-1.96)/0.86
	GA	64 (23.2)	103 (27.5)	1.29 (0.48-3.51)/0.61	0.14 (0.01-2.25)/0.16
	AA	8 (2.9)	8 (2.1)	Reference	Reference

†*p* values were adjusted for duration of hypertension, systolic blood pressure, cardiovascular disease, diabetic retinopathy, diabetic foot, HbA1c, serum-fasting glucose, serum-urea, serum-creatinine, serum-cystatin, urea-albumin/creatinine ratio (g/mol) - sample Number 1-3. OR: Odds ratio; CI: Confidence interval; HbA1c: Glycated hemoglobin; DN: Diabetic neuropathy

DECLARATION OF INTERESTS

The authors declare no conflict of interests

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