

ASSOCIATION BETWEEN POLYMORPHISM OF THE ANGIOTENSIN I CONVERTING ENZYME GENE AND HYPERTENSION IN TURKISH TYPE II DIABETIC PATIENTS

Mustafa Araz¹, Sükrü Aynacioglu², Sebnem Aktaran¹, Belgin Alasehirli², Vahap Okan¹

Gaziantep University, Medical Faculty, Gaziantep, Turkey: Department of Internal Medicine¹, Department of Pharmacology²

Summary: It has been suggested that an insertion/deletion (I/D) polymorphism in intron 16 of the angiotensin converting enzyme (ACE) gene may be associated with essential hypertension. The aim of this study was to examine the association between ACE I/D polymorphism with blood pressure level and hypertension status in Turkish type 2 diabetic subjects. Hundred and seven hypertensive (78 female, 29 male) and 132 normotensive type 2 diabetic subjects (73 female, 59 male) and 138 sex and age matched control subjects (87 female, 51 male) without diabetes and hypertension were included into the study. The I/D polymorphism was determined by polymerase chain reaction (PCR). There were no statistically difference in genotypic and allelic frequencies of the ACE I/D polymorphism between the hypertensive and normotensive diabetic patients and control subjects. Also no significant differences was detected in systolic and diastolic blood pressure among three different genotypes. ACE I/D polymorphism does not seem to play an important role in the development of hypertension in Turkish type 2 diabetic subjects, but prospective studies may show an association between ACE gene polymorphism and the development of hypertension in diabetic subjects.

Key words: *Angiotensin converting enzyme; Polymorphism; Type 2 diabetes mellitus and essential hypertension*

Introduction

The renin angiotensin system (RAS) is involved in the regulation of blood volume and pressure. RAS may have an important role in the pathogenesis of essential hypertension (EH). Angiotensin converting enzyme (ACE) is the key enzyme of RAS and contributes to the regulation of systemic and renal hemodynamics by converting angiotensin I to angiotensin II (8). Angiotensin II is a potent vasoconstrictor, a positive inotropic substance, a stimulus for aldosterone secretion and a growth promoter for heart and blood vessels (7,19,36,40).

EH is considered to be inherited as a polygenic trait and many genes have been suggested to be involved the etiology. One of the candidate genes that may be associated with EH is the ACE gene. A frequent insertion and / or deletion (I/D) polymorphism, characterised by the presence or absence of a 287 base pair fragment in intron 16 of the ACE gene has been identified (26,30). The individual variation of plasma ACE levels are mainly affected by this polymorphism and subjects that are homozygous for deletion (D/D), heterozygous (I/D) and those that are homozygous for (I/I) have highest, intermediated and lowest ACE plasma levels, respectively (29,38,42). Initially, an association between ACE gene polymorphism and EH and coronary

heart disease was suggested. Later, controversial results were reported for this association. It is known that the distribution of ACE genotypes shows ethnic differences (4,17). Therefore the significance of the ACE gene polymorphism in the pathogenesis of EH may differ between races.

Despite many reports for the association between ACE gene polymorphism and EH, data for diabetic subjects are restricted. Therefore, we studied the association between ACE I/D polymorphism with blood pressure level and hypertension status in Turkish type 2 diabetic subjects.

Material and Methods

Hundred and seven hypertensive (78 female, 29 male; mean age 56.6±7.9; mean duration of diabetes 8.9±6.2 years) and 132 normotensive type 2 diabetic subjects (73 female, 59 male; mean age 50.5±8.5; mean duration of diabetes 8.1±6.8 years) were included into the study. Age and sex matched 138 (87 female, 51 male; mean age 51.5±9.3; aged between 29-68) control subjects without diabetes and hypertension were selected randomly. Fasting plasma glucose levels of control subjects were less than 110 mg/dl and they had no family history of diabetes mellitus and essential hypertension in first degree relatives. Type 2 diabetes

mellitus was diagnosed according to the World Health Organisation criteria. Hypertensive group had been diagnosed in proportion as sixth report of the Joint National Committee (JNC) (37). A detailed family history was taken for each subject and all of them had a family history of essential hypertension in at least one parent and one sibling.

Normotensive diabetic subjects, without family history of essential hypertension in first degree relatives, were also selected according to JNC criteria. Informed consent according to the declaration of Helsinki as revised in 1996 was obtained from all patients. Duration of diabetes, BMI and blood pressure were recorded. Blood pressure was measured according to the sixth report of the JNC (37).

Serum fasting blood glucose, total cholesterol, triglyceride, HDL-cholesterol, serum and urine creatinine levels were determined by enzymatic methods (Olympus Diagnostica GmbH, Co.Clare, Ireland). LDL-cholesterol was calculated with Friedewald formula. HbA_{1c} was measured by HPLC (Bio Rad Diamat, München, Germany). Urinary albumin concentration was analysed by nephelometry (Behring Diagnostics GmbH, Marburg, Germany). According to urinary albumin/creatinine ratio ($\mu\text{g}/\text{mg}$) patients were classified as having normoalbuminuria (<30), microalbuminuria (30-300), and macroalbuminuria (>300). Multilevel control sera were included in each analytical run. Interassay and intrassay precision results were obtained from quality control data of the laboratory. Coefficient of variations was within 2.0-6.4%.

The I/D polymorphism was determined by polymerase chain reaction. Genomic DNA was extracted from leucocytes manually by the standard phenol/chloroform method and stored at +4°C until further analysis. The ACE I/D polymorphism was determined as described by Marre et al (20) with little modifications. Firstly, flanking primers GIIS (5'-CTCAAGCACGCCCTCACAGGACTG-3') and GAS (5'-GATGTGGCCATCACATTCGTCAGAT-3') were used to amplify intron 16 across the insertion. Amplification was carried out using Amplitaq DNA polymerase (Fermentas) with an initial incubation of 2 minutes at 94°C, followed by 15 cycles of 1 min at 94°C, 1 min at 62°C, and 1 min at 72°C in 25 ml buffer containing 2 mM MgCl₂ and 0.5% DMSO. Secondly, after cooling the samples briefly at 4°C, both the primer FYM (5'-ATCACGAGGT-CAGGAGATCGAGAC-3'), which corresponds to the insertion sequence, and GIIS were added into the tubes and PCR was continued for an additional 15 cycles in the same conditions with a final extension time of 7 min at 72°C. The extension between GAS and GIIS generated a 561-bp and a 274-bp PCR product for the I and D allele respectively. The addition of FYM and GIIS in the second step of the PCR created a 376-bp product, which is coexisting only with the I allele and increasing the sensitivity and specificity of insertion amplification.

Expected genotype frequencies were derived by the Hardy-Weinberg equation from single allele frequencies.

Genotype frequencies in patients and control groups were compared by the Chi-square test. Fisher's exact test was performed to compare allele frequencies between type 2 diabetic patients and control subjects. Values are presented as mean \pm SD. The relationship between ACE I/D genotypes and blood pressure in the patient group were evaluated by one-way analysis of variance (ANOVA). Student *t* test was used to compare the clinical and laboratory findings among patients with and without hypertension. All calculations were performed using the SPSS 6.0 program for Windows.

Results

The characteristics of the hypertensive and the normotensive subjects with type 2 diabetes mellitus were shown in Table 1. There were significant differences only on sex, BMI and LDL cholesterol between hypertensive and normotensive diabetic subjects. Eighty four per cent of patients were receiving ACE inhibitors.

Tab. 1: Characteristics of hypertensive and normotensive diabetic subjects.

Characteristics	Hypertensive (n:107)	Normotensive (n:132)	p
Sex (male/female)	78/29	73/59	0,01
Age (years)	56.6 \pm 7.9	50.5 \pm 8.8	NS
Diabetes duration (years)	8.9 \pm 6.2	8.1 \pm 6.8	NS
BMI(kg/m ²)	30.8 \pm 6.0	27.2 \pm 4.5	0.01
HbA _{1c} (%)	9.3 \pm 2.2	9.4 \pm 2.3	NS
Total cholesterol (mmol/L)	5.8 \pm 1.3	5.7 \pm 1.1	NS
LDL-cholesterol (mmol/L)	3.7 \pm 1.1	3.4 \pm 0.9	0.04
HDL-cholesterol (mmol/L)	1.2 \pm 0.4	1.2 \pm 0.4	NS
Trygliceride (mmol/L)	2.5 \pm 1.7	2.7 \pm 0.2	NS

All statistical comparisons were performed with student-t test, except comparison according to sex, which was calculated by Chi-square test.

Observed genotype and allele frequencies of the ACE I/D polymorphism in patient and control group are given in Table 2. Expected genotype frequencies were in good agreement with the Hardy-Weinberg equation. The most frequent genotype was ID in patient and control groups followed by the DD and II genotype respectively. There were no statistically difference in genotypic and allelic frequencies of the ACE I/D polymorphism between the hypertensive and normotensive diabetic patients and control subjects.

When the allele frequencies observed in hypertensive subjects were compared according to sex, also no significant difference was found (D:I (%), male; 61.5:38.5, female; 62.1:37.9).

When clinical and laboratory characteristics of the total diabetic patients were compared among three different genotypes, no significant differences was detected in systolic and diastolic blood pressure, urinary albumin excretion rate and the other characteristics (Table 3). There was also no significant difference in genotype distribution among ACE inhibitor using patients with and without nephropathy.

Tab. 2: Frequencies of ACE genotypes and alleles among hypertensive, normotensive and control group.

	Hypertensive n (%)	Normotensive n (%)	p	Controls n (%)
<i>Genotype</i>				
DD	32 (29.9)	47 (35.6)	NS	52 (37.7)
ID	55 (51.4)	65 (49.2)	NS	65 (47.1)
II	20 (18.7)	20 (15.2)	NS	21 (15.2)
<i>Allele</i>				
D	59 (55.1)	80 (60.6)	NS	169 (61.2)
I	48 (44.9)	52 (39.4)	NS	107 (38.8)

Tab. 3: Characteristics of diabetic patients according to ACE genotype.

Variable	DD (n=77)	ID (n=121)	II (n=41)	P
Sex (male/female)	32/45	43/78	13/28	NS
Age (years)	53±8	53±9	54±10	NS
Diabetes duration (years)	8.5±6.1	8.2±6.7	8.8±7.3	NS
BMI (kg/m ²)	28.8±5.6	29.4±5.3	29.5±6.6	NS
SBP (mmHg)	145±29	141±24	139±28	NS
DBP (mmHg)	89±14	84±12	85±13	NS
HbA _{1c} (%)	9.5±2.2	9.4±2.2	9.3±1.8	NS
Total cholesterol (mmol/L)	5.6±1.2	5.9±1.2	5.6±1.2	NS
LDL cholesterol (mmol/L)	3.5±0.9	3.6±1.0	3.4±1.0	NS
HDL cholesterol (mmol/L)	1.2±0.4	1.2±0.4	1.1±0.4	NS
Triglyceride (mmol/L)	2.4±1.5	2.8±2.3	2.4±1.1	NS
Normoalbuminuria (%)	55.8	47.1	56.0	NS
Microalbuminuria (%)	19.5	24.8	22.0	NS
Macroalbuminuria (%)	24.7	28.1	22.0	NS
NS,	non-significant			

Discussion

EH is a multifactorial and polygenic disease that results from an interaction between genetic and environmental factors (28). RAS plays a major role in blood pressure regulation. The genes of RAS are attractive candidate genes for the association with EH and one of these is the ACE gene. Although the I/D polymorphism in the ACE gene mainly affect plasma ACE levels, there is controversial results regarding the association of this polymorphism with blood pressure and hypertension. No significant association was reported in the majority of the previous studies (3,5,10,12, 13,15,16,18,21,34,41). However, D allele, or DD genotype was found to be related with EH in some studies (1,6,44). The conflicting results of association studies for EH may be explained by the different genotype and allele distribution in different populations, which shows marked interracial and interindividual variation (4,17). For example, the less frequent genotype in healthy Caucasian European and American population is II (4,35), whereas in healthy Japanese and Chinese populations DD (24,43). In our study, the less frequent genotype in healthy and diabetic subjects

were also II like other Caucasian populations. DD genotype is reported as the most frequent genotype in most Caucasian populations, but the most frequent genotype was ID in our study group. There was also no statistically significant difference in allele frequencies and genotype distribution among the control and diabetic group. Although ethnic differences may be an explanation for the controversy, conflicting results were also reported in the same populations (13,15,22).

In this study, no significant association between ACE I/D polymorphism and hypertension in Turkish diabetic subjects was found similar to the majority of the previous studies with EH in non-diabetic subjects (3,5,10,12,13,15, 16,18,21,34,41). Despite there are many reports for the association between ACE I/D polymorphism and EH in non-diabetic subjects, data for diabetic subjects is restricted. Pujia et al (27) first reported an association between ACE DD genotype and hypertension in type 2 diabetic patients in a small group. Bengtsson et al (2) also reported that the DD genotype was associated with HT in Swedish type 2 diabetic patients. Ukkola et al (39) also found a relation between ACE I/D polymorphism and hypertension in type 2 diabetic patients, but they suggested a lower systolic blood pressure level and prevalence of hypertension with II genotype. In contrast, Huang et al (14) found no difference in blood pressure of Finn type 2 diabetic subjects among the genotypes similar to our results. We studied the possible association between ACE I/D polymorphism and EH in Turkish type 2 diabetic patients and found also no association either with blood pressure or with hypertension status. Similarly to the majority of the previous studies within Caucasian type 2 diabetic populations (9,11,25,31,33) our findings did not show an association between ACE genotype distribution and nephropathy status.

Some authors also suggest a gender relationship of D allele or DD genotype with EH (23,32). Also a contradiction exists for this gender relationship. Donnel et al (23) suggest that the DD genotype was related with an increased diastolic blood pressure in men, however Sagnella et al (32) suggest that the D allele showed a highly significant association with EH in women.

In conclusion, these all controversial results suggest that the association between ACE I/D polymorphism and blood pressure is more complex. The I/D polymorphism of the ACE gene seems not to play an important role in the development of hypertension in Turkish type 2 diabetic subjects, but prospective studies may determine the development of hypertension according to ACE genotype in normotensive diabetic subjects.

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Dr. Mustafa Araz,
Gaziantep University, Medical Faculty,
Department of Internal Medicine,
TR-27070 Gaziantep,
Turkey.
e-mail: araz@gantep.edu.tr