Angiotensin-I converting enzyme gene polymorphism in Turkish type 2 diabetic patients

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Abbreviations: ACE, angiotensin-I converting enzyme; BMI, body mass index; DM, diabetes mellitus; PCR, polymerase chain reaction; RAS, renin angiotensin system

Abstract

Non-insulin dependent diabetes mellitus is often associated with some complications such as nephropathy, retinopathy and neuropathy. Genes of the renin angiotensin system are potential candidate genes for diabetic complications. We investigated the relationship between angiotensin converting enzyme (ACE) gene polymorphism in type 2 diabetic patients with and without diabetic nephropathy. Seventy five patients (25 type 2 diabetic patients with nephropathy, 50 type 2 diabetic patients without nephropathy) and 37 healthy controls were studied. Gene polymorphism of ACE was determined by PCR (polymerase chain reaction) amplification using allele-specific primers. The frequencies of ACE DD, ID and II genotypes among the patients with type 2 diabetic patients were found 48%, 42%, 10% whereas in control subjects, 27%, 60%, 13% respectively. Type 2 diabetic patients carrying DD genotype without nephropathy increased 1.77 fold than control subjects ($P < 0.05$). There is no significant correlation between diabetic nephropathy and ACE gene polymorphism. But we found that ACE DD genotype increased significantly in type 2 diabetic patients com-

pared to control subjects ($P < 0.05$).

Keywords: ACE genotypes; diabetic complications; diabetes mellitus type 2; diabetic nephropathy; renin angiotensin system

Introduction

Non-insulin dependent diabetes mellitus is the most common type of diabetes. Patients with diabetes mellitus (DM) have risk of developing some complications such as diabetic nephropathy, diabetic retinopathy and cardiovascular disease. Especially, genetic factors, poor glycemic control and hypertension may play role in the diabetic complications coming up (Sequist *et al.*, 1989; Freire *et al.*, 1994). Diabetic nephropathy is an important complication of diabetes mellitus of both type1 and type2 and is observed in up to 30% of patients, who suffer from non-insulin dependent diabetes mellitus (Nelson *et al.*, 1991; Chowdhury *et al.*, 1999). Studies on the genes have proposed the renin angiotensin system (RAS) as an important genetic factor for diabetic complications. Renin angiotensin system may play an important role in blood pressure regulation and acts a key regulator of sodium homeostasis. One of the RAS genes is gene encoding the ACE, which may be associated with diabetic nephropathy. Angiotensin-I converting enzyme (ACE) or kininase II, is a dipeptidyl carboxypeptidase which plays an important role in blood pressure regulation and electrolyte balance by hydrolyzing angiotensin I into angiotensin II which is a potent vasopressor, and aldosterone-stimulating peptide. The enzyme is also able to inactivate bradykinin which is a potent vasodilator. Angiotensin-I converting enzyme gene has 26 exons; exons 1-12 encode for the amino domain, exons 13-26 encode for the carboxyl domain. The gene has been described with an insertion and deletion polymorphism (I/D) of a 287bp *Alu* repetitive sequence in intron 16 (Rigat *et al.*, 1990). Therefore three genotypes were found (DD, II homozygotes and ID heterozygote). This polymorphism accounts for a large proportion of the variability of serum ACE activity (for DD genotypes having the highest and II genotypes having the lowest ACE activity) (Rigat *et al.*, 1990). Additionally, there is also an association between the D allele and higher plasma levels of ACE, which also seems to be associated with diabetic complications (Marre *et al.*,

1994; Marre *et al.*, 1997). Prior studies have shown that angiotensin converting enzyme inhibitors (ACEi) can impede with the progress of improving diabetic nephropathy (Orisio, 1999; Ha *et al.*, 2000). Angiotensin-I converting enzyme is not only effective in playing a role in nephropathy, but also participates in cardiac complications and hypertension (Bedir *et al.*, 1999; Estacio *et al.*, 1999; Donnelly *et al.*, 2000). According to previous studies, there are ethnic differences in the frequencies of I/D polymorphism in different ethnic groups (Barley *et al.*, 1994; Doi *et al.*, 1996; Gutierrez *et al.*, 1997; Sagnella *et al.*, 1999; Fernandez, 2000). In this study, the relationship between polymorphisms of ACE gene and diabetic nephropathy was investigated in Turkish type 2 diabetic patients.

Materials and Methods

Patients selection and clinical investigation

ACE gene polymorphism was studied in 75 patients (25 type 2 diabetes with Diabetic nephropathy, 50 type 2 diabetes without Diabetic nephropathy) and 37 healthy controls. Diabetic patients were recruited from Turkish Diabetes Association, Harbiye Hospital of Diabetes. A detailed medical history of each patients was obtained.

All reagents were analytical grade unless otherwise stated. EDTA, sodium dodecyl sulphates, primers, dNTP set and *Taq* DNA polymerase were obtained from Sigma Chemical Co, Boehringer Mannheim and MBI Fermentase, respectively.

Blood pressure was measured as recommended by the American Association (Kirkendall *et al.*, 1980). The subject lay supine for 10 min and afterwards the blood pressure was measured with a mercury sphygmomanometer. The readings were taken from the left and the right arms and recorded to the nearest 2 mmHg and the mean was calculated. The weight and the height were recorded and the body mass index was calculated using the formula BMI: weight/height² (kg/m²). Fasting blood samples were collected to determine blood glucose (Zaloga, 1997) and urea (Bauer *et al.*, 1974) levels. Creatinine concentrations were determined by the automated method of Bartels (Bartels *et al.*, 1972).

The presence or absence of myocardial infarction was documented by the electrocardiography (ECG).

Nephropathy was defined as the presence of macroalbuminuria (>300 mg/day) and serum urea (>50 mg/dl) and creatinine (>1 mg/dl) levels.

Retinopathy was defined by funduscopy and neuropathy was defined by physical examination and electromyography (EMG) by neurology specialists. History of diabetes was also recorded.

Healthy persons without any symptoms of diabetes were selected for the control group.

Isolation of DNA

Blood specimens were collected in tubes containing EDTA and DNA was prepared from the leukocyte pellets by sodium dodecyl sulphate lysis, ammonium acetate extraction and ethanol precipitation (Miller *et al.*, 1988).

Genotyping method for ACE I/D Polymorphism

Template DNA (0.5-1.0 µg) was used in a PCR under stringent conditions to avoid the possibility of false positives for ACE genotyping. Reactions were performed with 10 pmol of each primer: forward primer 5'-CTGGAGACCACTCCCATCCTTCT-3', reverse primer 5'-GATGTGGCCATCTTCGTCAGAT-3' in final volume of 50 µl containing 3 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl (pH 8.4), 0.5 mM of each dNTP (MBI Fermentas) and 1 unit *Taq* Polymerase (MBI Fermentas). Amplification was carried out in a DNA Thermal Cycler (MJ Research Techne) for 30 cycles with denaturation steps at 94°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 2 min (Rigat *et al.*, 1992). PCR products were separated on 3% agarose gel and DNA was visualized by ethidium bromide staining. The DNA product is a 190 bp fragment in the presence of the deletion (D) allele and a 490 bp fragment in the presence of the insertion (I) allele. Thus, there are three genotypes after electrophoresis: a 490 bp band (genotype II), a 190 bp band (genotype DD), or both 490 and 190 bp band (genotype ID).

Each sample which had the DD genotype was submitted to PCR amplification with a primer pair (5'-TGGGACCACAGCGCCCGCCACTAC-3' and 5'-TCGCCAGCCCTCCCATGCCCATAA-3') that recognizes an insertion specific sequence. If the DNA was mistyped, a PCR product of 335 bp is present (Shanmugam V *et al.*, 1993). The 335 bp fragment was identified on 2% agarose-gel containing ethidium bromide.

Statistical analyses

Frequency labels and statistical analysis were performed with SPSS 7.5 for Windows. Data were presented as mean ± standard deviation (SD) or as proportions. A double sided *P* value of 0.05 was defined as statistically significant. Expected and observed frequencies of genotypes were compared with χ^2 analysis.

Results

Clinical investigation

The study groups were compared with respect to biochemical parameters (Table 1). As nephropathy markers serum urea and creatinine levels increased significantly in patients with nephropathy compared to those without nephropathy and control groups. ($P <$

Figure 1. The homozygous individuals for the D allele (DD genotype) were identified by the presence of a single 190 bp product. The homozygous for the I allele (II genotype) were identified by the presence of a single 490 bp product. The heterozygous individuals (ID genotype) were identified by the presence of both 190 bp, and 490 bp products.

0.05). And also fasting glucose levels were significantly higher in type 2 diabetes with and without nephropathy compared to that in control group ($P < 0.001$).

As shown in Table 2, we did not observe any significant difference between type 2 diabetic patients with and without nephropathy for duration of diabetes. Also we did not find any association between frequencies of the presence of retinopathy, neuropathy and cardiac events in patients with and without nephropathy in our study. Systolic and diastolic blood pressure were higher in patients with and without nephropathy compared to control group. Besides, systolic blood pressure levels elevated significantly in type 2 diabetes with nephropathy compared to those without ($P < 0.05$).

Frequencies of ACE polymorphism

Table 2 and Figure 1 show the distribution of ACE gene polymorphism in study groups. The frequencies of ACE DD, ID and II genotypes among the patients in type 2 diabetic patients were 48%, 42%, 10%, in control subjects 27%, 60%, 13% and in patients with nephropathy 36%, 44%, 20%, respectively. We observed the ones carrying DD genotype in type 2 diabetic patients 1.77 times ($\chi^2 = 7.85$ $P = 0.005$, OR: 1.77, 95%CI: 1.160-2.720) increased than control

Table 1. Biochemical parameters in patients and control group.

Groups	Type 2 diabetes without nephropathy (n = 50)	Type 2 diabetes with nephropathy (n = 25)	Control (n = 37)
Urea (mg/dl)	36.9 ± 11.1*	56.5 ± 22.7*	< 50
Creatinine (mg/dl)	0.9 ± 0.15*	1.08 ± 0.38*	< 1.00
Fasting Blood Glucose (mg/dl)	171.0 ± 56.0*	180.7 ± 53.8*	84.3 ± 11.4*

*Data are expressed as means ± SD. n: Number of individuals

Table 2. Clinical characteristics in patients and healthy control group.

Groups	Type 2 diabetes Without nephropathy (n = 50)	Type 2 diabetes with nephropathy (n = 25)	Control (n = 37)
n (Female/Male)	33/17	20/5	15/22
Age (Years)	57.1 ± 14.5*	59.6 ± 13.5*	52.1 ± 16.3*
Duration of diabetes (Years)	13.0 ± 9.2*	14.6 ± 10.4*	-
Body mass index (kg/m ²)	26.1 ± 4.2*	26.3 ± 3.9*	24.7 ± 3.7*
Systolic blood pressure (mmHg)	133.9 ± 21.1*	143.8 ± 16.7*	121.2 ± 9.4*
Diastolic blood pressure (mmHg)	84.4 ± 12.9*	85.8 ± 12.9*	75.3 ± 8.0*
Patients with retinopathy (%)	22	16	-
Patients with neuropathy (%)	16	8	-
Patients with cardiac events (%)	20	24	-

*Data are expressed as means ± SD. n: Number of individuals.

Table 3. Distribution of angiotensin converting enzyme (ACE) genotype and allele frequencies in study groups.

Genotypes	Type 2 diabetes with Nephropathy (n = 25)		Type 2 diabetes without Nephropathy (n = 50)		Control (n = 37)	
	n	%	n	%	n	%
DD	9	36	24	48	10	27
ID	11	44	21	42	22	60
II	5	20	5	10	5	13
Allels						
D	29	58	69	66	42	57
I	21	42	31	34	32	43

Data were compared between groups by χ^2 test. n: number of individuals

subjects.

Discussion

We examined ACE gene polymorphism, one of the important genes in renin angiotensin system, in diabetes mellitus patients and healthy control group in Turkey. Genetic factors, lipid profiles, hypertension are potential risk factors to improve the complications in diabetes (Katsuya *et al.*, 1995; Hanssen, 1997). Renin Angiotensin system should modulate a risk for diabetic complications. A meta analysis about the association between I/D polymorphism of ACE and nephropathy was published in 1998 by Fujisawa *et al.* Yoshida *et al.* divided patients with type 2 diabetes into two groups. First group had a stable renal function, second group had a declining renal function. They showed that ACE genotypes revealed that the majority (95%) of patients with the DD genotype who had albuminuria progressed to end-stage renal disease within 10 years of diagnosis of diabetes (Yoshida *et al.*, 1997). Jeffers *et al.* showed that an association between ACE DD genotypes and diabetic nephropathy (Jeffers *et al.*, 1997). There are also previous reports that show no association between ACE genotypes and diabetic nephropathy. Schmidt *et al.* found that no differences between ACE allele and genotype frequency and type 2 diabetic patients with and without nephropathy in 1997 (Schmidt *et al.*, 1997). Liao *et al.* did not observe that any association between ACE genotypes and diabetic nephropathy in type 2 diabetic patients (Liao *et al.*, 1999). Also we did not find any association between ACE genotypes and diabetic nephropathy. We observed that the frequencies of ACE DD, ID and II genotypes between the patients with type 2 diabetes 48%, 42%, 10% and patients with nephropathy were 36%, 44%, 20% respectively. The findings of the our study agree with some studies (Jeffers *et al.*, 1997; Schmidt *et*

al., 1997; Liao *et al.*, 1999). There is no difference between patients with nephropathy and other study groups in ACE alleles and genotypes frequencies in our study.

Our data also showed an association between ACE DD genotypes and Type 2 diabetes. We found that carrying DD genotype in type 2 diabetic patients without diabetic nephropathy 1.77 times increased than control subjects ($P < 0.05$) suggesting that the DD genotype is associated with an increased susceptibility to type 2 diabetes in our study population. The results show that decreasing of the carrying DD genotypes in type 2 diabetic patients means that ACE DD genotype may play an important role in development of diabetes. Our study appears to be similar to some previous reports. Hsieh *et al.* studied type 2 diabetic patients with and without nephropathy and healthy control group and showed that carrying DD genotype increased than healthy controls (Hsieh *et al.*, 2000). Feng Y *et al.* showed that The DD frequency was significantly higher in type 2 diabetic patients than in the control group (Feng *et al.*, 2002). A meta analysis revealed that the risk of nephropathy was increased in the presence of DD or ID genotype in Asian patients with type 2 diabetes but not in Caucasian patients (Kunz *et al.*, 1998).

No association was found between frequencies of the alleles and the presence of retinopathy, neuropathy and cardiac events in patients with and without nephropathy in our study.

There are several possible reasons for the discrepancy between the previous studies and our study. First it might be due to ethnic differences or sampling bias. Second the background of the study subjects recruited from a diabetes clinic in our selected population may have played a role. Racial differences including diverse social or cultural factors might have also contributed to the results.

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