

## Association of Angiotensin Converting Enzyme (ACE) Gene Polymorphism and Diabetic Nephropathy

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### Abstract

Angiotensin I-converting Enzyme (ACE) gene polymorphism; genotype DD or D allele may be involved with an increased susceptibility to type 2 diabetes and diabetic nephropathy (DN). We examined the frequency of ACE gene polymorphism in 170 patients (85 type 2 diabetes with nephropathy and 85 without it) in Tehran, Iran. DNA was extracted from the white blood cells and the I/D polymorphism of the ACE gene was detected by PCR. The frequency of DD, ID and II genotypes in type 2 diabetic patients were 20%, 61.2% and 18.8%, and in patients with nephropathy 30.6%, 55.3%, 14.1%, respectively. The DD genotype of the DN group was higher than that of the type 2 diabetes patients (30.6% vs 20%,  $P=0.157$ ,  $RR=1.3$ ) and the control group (30.6% vs 14.3%,  $P=0.006$ ,  $RR=2.9$ ). The frequency of D allele in nephropathic patients was 58.2% as compared to the type 2 diabetic patients without nephropathy (50.5%)  $P=0.19$ ,  $RR=1.16$ . The D allele frequency in the DN group was found slightly higher than of the type 2 diabetes ( $X^2=0.684$ ,  $OR=0.709$ , 95%CI: 0.313-1.606,  $P=0.408$ ) which indicated the D allele was not associated with DN. It is suggested that DD genotype and D allele are not associated with diabetic nephropathy.

**Keywords:** Angiotensin converting enzyme, Genetic polymorphism, Insertion/deletion, Diabetic nephropathy, Iran

### Introduction

Diabetic nephropathy (DN) is now the most common cause of end stage renal disease (1, 2). Patients with diabetes mellitus (DM) have the risk of development some complications such as diabetic nephropathy (DN), diabetic retinopathy and cardiovascular disease. Despite numerous reports suggesting a substantial genetic contribution to the susceptibility to type 2 diabetes (T2D) and diabetic nephropathy, no major susceptibility genes have been identified so far (1, 2). ACE plays an important role in the renin-angiotensin system cascade by converting angiotensin I to angiotensin II (3-7). Angiotensin II (Ang II) is a potent vasoconstrictor of the systemic and the local blood pressure (8-12). Ang II increases systemic and glomerular blood

pressure, stimulates mesangial cell proliferation and tissue growth (13- 15).

Some of studies suggest that genetic factors may be involved in the etiology of renal disease in T2D (16-20). One possible genetic factor is the angiotensin-I converting enzyme (ACE) gene insertion (I), deletion (D) polymorphism within the human ACE gene (21-23).

Based on the presence or absence of a 287 base pair sequence in intron16, three genotypes DD, II homozygotes and ID heterozygote are found (24-27). About the association of the ACE and ID polymorphism and the development of diabetic nephropathy, Staessen et al. have reported a general increased risk for diabetic nephropathy in individuals of carrying the D allele (28).

Results from recent morphological studies in patients with T2D have verified this hypothesis that the DD genotype is associated with progression of DN (29-32). In contrast, Kunz et al. did not observe this association (33).

The goal of the present study was to investigate the relationship of ACE gene polymorphism with increasing risk of nephropathy in patients with T2D in Tehran (capital of Iran) population.

## Materials and Methods

We studied ACE gene polymorphism in 170 patients (85 T2D with nephropathy and 85 without it). T2D patients were recruited from Imam Hospital of Tehran University of Medical Sciences, and then a detailed medical history of each patient was obtained. Blood pressure was measured as recommended by the American Diabetic Association.

The weight and the height were recorded and the body mass index (BMI) was calculated using the formula BMI: Weight/ Height ( $\text{kg}/\text{m}^2$ ). Nephropathy was defined as the presence of macroalbuminuria ( $\geq 300$  mg/day).

After a 12 h overnight fasting, 10 ml of 15% EDTA anticoagulated blood samples and serum were drawn from the patients, and centrifuged within 2 h. Buffy coat layers for DNA extraction and serum were refrigerated at  $-20^\circ\text{C}$ .

Urinary excretion of albumin was measured by 24 h urine collection.

Genomic DNA was isolated from peripheral blood leukocytes according to a standard salting out method (34). Amplification was carried out in a DNA thermocycler [Eppendorf Master cycler]. First, PCR was performed using 20 pmoles of each primer (flanking primer pair):

Sense oligo 5' - CTG GAG ACC ACT CCC ATC CTT TCT 3' and anti sense oligo:

5' -GAT GTG GCC ATC ACA TTC GTC AGA T-3' in a final volume of 25  $\mu\text{l}$ , containing (0.5  $\mu\text{g}$  genomic DNA, 2 mM  $\text{MgCl}_2$ , 50 mM  $\text{KCl}$ , 10mM Tris- Hcl (pH=8.3), 0.2 mM of each dNTP, and 0.5 unit of Taq polymerase. PCR was done with an initial denaturing time at  $94^\circ\text{C}$  for 1 min.

Then the DNA was amplified for 30 cycles with denaturation at  $94^\circ\text{C}$  for 30 s, annealing at  $58^\circ\text{C}$  for 30 s, and extension at

$72^\circ\text{C}$  for 1 min. This was followed by final extension at  $72^\circ\text{C}$  for 8 min.

PCR products were directly visualized using ethidium bromide staining after electrophoresis in a 2% agarose gel (35-37).

The amplification 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.

Therefore, there were three genotypes after electrophoresis:

A 490 bp band (genotype II), 190 bp band (genotype DD), or both 490 and 190bp band (genotype ID). Mistyping of ID heterozygote as D homozygotes may occur.

Thus, each sample that had the DD genotype was applied to PCR amplification using the forward:

5- TCG GAC CAC AGC GCC CGC CAC TAC-3' ; and the reverse:

5' - TCG CCA GCC CTC CCA TGC CCA TAA-3' primers with identical PCR conditions except for an annealing temperature of  $67^\circ\text{C}$ .

The reaction yields a 335-bp amplicon only in the presence of an I allele, and no product for homozygous DD samples.

Urinary albumin was measured by immuno turbidometry method (CECIL9000, USA). HbA1c was measured by HPLC method (DSS Modle, DREW-England).

**Statistical analysis** The SPSS statistical software package version 11.5 was used for the statistical analyses. Genotype and allele frequencies of ACE gene polymorphism were compared between type 2 diabetic patients with and without nephropathy using  $\chi^2$ -test. Odds ratios (OR) as estimates of relative risk for disease were calculated with 95% confidence intervals. Two-tailed Student's *t*-test was also used to compare quantitative data. Statistical significance was assumed at the  $P < 0.05$  level.

## Results

The ACE gene frequencies are presented in Table 3 and Fig. 1.

As shown in Table 1, T2D patients with and without nephropathy were well-matched for gender, age, body mass index (BMI), duration of diabetes, biochemical parameters and HbA1c values. The average age of the control subjects (59.5±7.6 yr) was slightly higher than that of the DN (59.2-8.2), but gender distribution was the same in both groups ( $X^2=1.6$ ,  $df=1$ ,  $P=1.000$ ).

Nephropathic patients had significantly higher systolic blood pressure values. The study groups were compared with respect to clinical characteristic and biochemical parameters. Serum creatinine, fasting blood glucose (FBS) levels and urinary protein/day increased significantly in patients with nephropathy compared to those without it ( $P<0.05$ ). Table 2 shows that the patients clinical and biochemical characteristics did not vary according to ACE genotype status.

**Table 1:** Characteristics of type 2 diabetic patients with and without nephropathy

Variables	Type 2 diabetic patients	Type 2 diabetic patients with	*P Value
	without nephropathy (control) n=85	nephropathy(case) n=85	
Age ( yr)	59.5±7.6 <sup>a</sup>	59.2-8.2	0.802
Sex (M/F)	43/42	43/42	1.000
Diabetic duration (yr)	14.5±3.6	11.6-4.7	0.000
BMI (kg/m <sup>2</sup> )	27.9±12.8	26.4-3.9	0.282
Systolic Blood Pressure mmHg	134.8±17.6	140.7-26.1	0.091
Diastolic Blood Pressure mmHg	86.2±11.6	89.1-10.2	0.107
FBS (mg/dl)	204.6±52.1	226.4-63.3	0.015
Cholestrol (mg/dl)	207.2±39.4	217.8-45.7	0.110
Triglyceride (mg/dl)	199.2±92.9	198.7-88.4	0.973
HDL (mg/dl)	43.6±9.5	42.9-9.5	0.669
LDL (mg/dl)	123.1±35.5	121.9-31.2	0.815
VLDL (mg/dl)	37.4±10.9	36.9-13.3	0.806
Creatinine (mg/dl)	0.89±0.19	1.05-0.25	0.000
HbA1c (%)	8.3±1.9	8.8-1.9	0.146
Urinary protein (mg/day)	6.46±3.7	800.1-393.6	0.000
Urinary volume (ml/day)	1440.8±332.9	1374.1-609.2	0.377

a.Data are presented as mean±SD. Comparisons were made using student's *t* test (for continuous variables). Statistically significant; \* $P<0.05$

**Table 2:** Clinical and biochemical characteristics of the subjects distributed according to their ACE genotype

Variable	DD	ID	II	P*
Age( yr)	59.6±8.47	59.5±7.97	58.2±6.9	0.891
Sex (M/F)	26/25	43/48	17/11	0.459
Diabetic duration (yr)	12.2±4.65	13.41±4.08	13.3±5.15	0.103
BMI (kg/m)	26.8±4.75	27.9±12.1	25.4±5.46	0.499
Systolic (mmHg)	140.5±20.1	136.72±23.12	136.10±24.10	0.599
Diastolic (mmHg)	88.9±11.10	87.30±23.12	86.46±10.40	0.656
FBS (mg/d)	224.33±68.36	210±51.79	216.18±61.71	0.527
Cholesterol (mg/d)	219.84±44.45	208.87±40.72	211.1±46.37	0.361
Triglyceride (mg/dl)	221.63±105.1	194.24±82.9	173.11±77.8	0.541
HDL (mg/d)	43.71±9.65	43.59±9.81	41.61±7.94	0.703
LDL (mg/dl)	124.53±36.1	123.4±32.1	115.6±32.5	0.123
VLDL (mg/dl)	40.1±12.8	36.6±12.1	33.64±10.0	0.769
Urinary protein (mg/day)	592.06±548.21	305.70±421.03	376.7±474.3	0.59

Data are presented as mean± SD. Comparisons were made using Student's *t*-test (for continuous variables). Statistically significant; \**P*< 0.05 between DD and ID/II

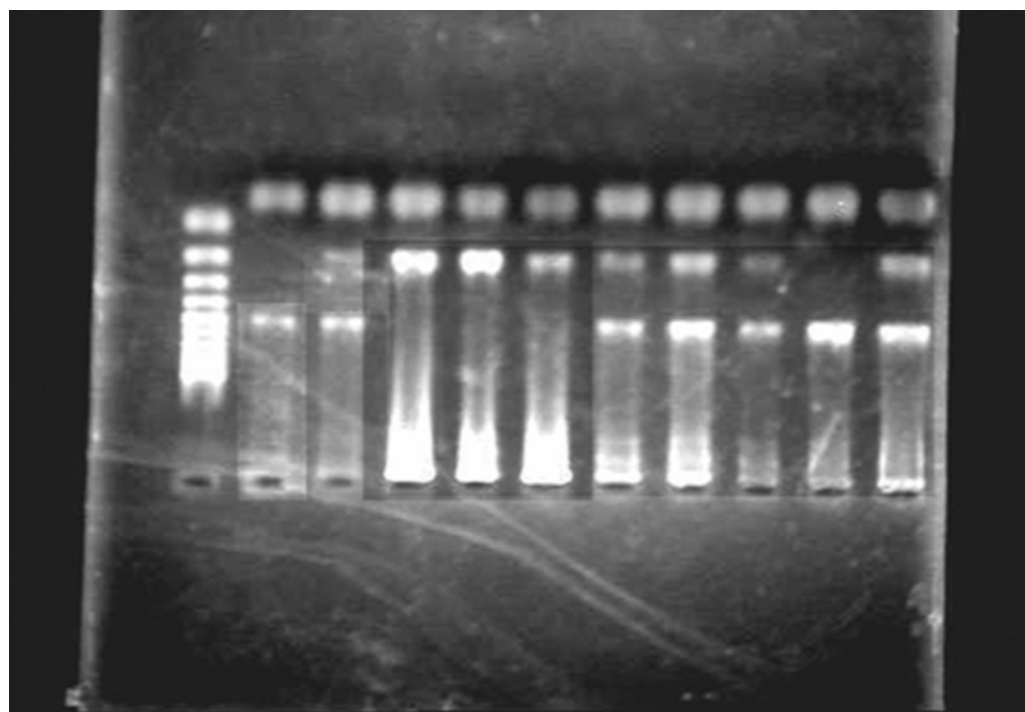
**Table 3:** Allele and genotype frequencies of gene insertion /deletion polymorphism in T2D with and without nephropathy

Genotype	DN* n=85		DM** n=85		P value Type 2 DM vs Type 2 diabetes with DN
	n	%	n	%	
DD	26	30.6	17	20	<i>P</i> = 0.157, RR= 1.3
ID	47	55.3	52	61.2	<i>P</i> = 0.53, RR= 0.8
II	12	14.1	16	18.8	<i>P</i> = 0.53, RR= 0.83
allele					
D	99	58.2	86	50.5	<i>P</i> = 0.19, RR= 1.16
I	71	41.7	84	49.4	<i>P</i> = 0.19, RR= 0.85

The distribution and comparison of alleles and genotypes frequency of ACE gene polymorphism in each was made using Chi-square test, Fisher,s exact, likelihood ratio

\*DN; Diabetic nephropathy

\*\*DM; Type 2 diabetes mellitus



190 bp band  
490 bp band

**Fig. 1:** Detection of ACE I/D polymorphism M, 100-1000 bp DNA ladder; DD homozygous: a single 190 bp product; ID heterozygous: both 190 bp and 490 bp; II homozygous: a single 490 bp product.

## Discussion

Although the I/D polymorphism is in the intronic region of the ACE gene, many studies showed that the DD genotype strongly was associated with increased serum ACE levels. The DD genotype leads to higher ACE expression and activity and may be predispose individuals to T2D and its complications (5). We examined ACE gene polymorphism, one of the important genes in Renin-Angiotensin system (RAS), in T2D patients with and without nephropathy.

There are plausible reasons to support an association between ACE genotype and diabetic nephropathy. Clinical trials have shown that antihypertensive treatment decreases proteinuria in hypertensive diabetic patients with nephropathy (19, 20). A meta analysis showed that the risk of nephropathy was increased in the presence of DD or ID genotypes in Asian patients with types diabetes (16). Jeffers et al. have shown an association between ACE DD genotype and DN (38).

Schmidt et al. found no differences between ACE allele and genotype frequency in diabetic patients with and without nephropathy (39). Liao et al. did not observe any association between ACE genotypes and DN (40).

Our study did not show a positive association between the DD genotypes of ACE gene polymorphism in DN and type 2 diabetic. Relative risk for DD homozygous subjects was 1.30 (DN vs DM, 95% CI= 0.95-1.70,  $P= 0.15$ ).

We also investigated the frequency between the D allele (DD+ID) and II genotype in two groups. The D allele frequency in DN group was found slightly higher than that of the T2D ( $X^2= 0.684$ , OR= 0.709, 95% CI: 0.313-1.606,  $P= 0.408$ ) that indicated the D allele was not associated with DN. The observed genotype distribution was in agreement with the Hardy-Weinberg proportion.

We also investigated the relationship between the D allele and the development of DN in T2D patients with nephropathy (95% CI: 0.93-1.45,

RR= 1.16,  $P= 0.19$ ). Our study showed that the D allele did not associate with DN. Our finding is not in accordance with data obtained in the report of Ohno (Odds ratio= 2.6) and Yoshida (Odds ratio= 4.6) (41, 42), but this study is consistent with previous studies in Asian population (43-49).

In conclusion, data of this investigation concluded that DD genotype and D allele in type 2 diabetic patients with and without nephropathy were insignificant.

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