

# Angiotensin Converting Enzyme Gene Polymorphism in Iranian Patients with Type 2 Diabetes

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## ABSTRACT

**Background:** Angiotensin I converting enzyme (ACE) is a Zinc metalloproteinase, converts Ang-I to Ang- II, a pro-inflammatory agent which may contribute to pathophysiology of some diseases like type 2 diabetes. **Objective:** To investigate the relationship between ACE I/D polymorphism and type 2 diabetes in 261 Iranian case-control pairs. **Methods:** 170 patients (85 type 2 diabetics with nephropathy and 85 type 2 diabetics without nephropathy) and 91 healthy control subjects were enrolled in our study. I/D polymorphism of the ACE gene was detected by polymerase chain reaction (PCR) utilizing specific primers. **Results:** The frequency of DD genotype in the DN group was higher than that of the type 2 diabetic patients (30.6% vs. 20%,  $P=0.157$ ) and the control group (30.6% vs. 14.3%,  $P=0.006$ ). The frequency of D allele in nephropathic patients was 58.2% as compared to type 2 diabetic patients without nephropathy 50.5% ( $P=0.19$ ) and control subjects 37.3% ( $P=0.001$ ). Therefore, the frequency of DD genotype and D allele significantly increased in DN patients in comparison to healthy controls. **Conclusion:** It is concluded that the DD genotype and/or D allele of ACE gene may increase the risk for type 2 diabetes but not diabetic nephropathy.

**Keywords:** Angiotensin Converting Enzyme, Type 2 Diabetes, Nephropathy

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## INTRODUCTION

Type 2 diabetes represents a significant health problem worldwide. Patients with diabetes mellitus (DM) have the risk of developing complications such as diabetic nephropathy (DN), diabetic retinopathy and cardiovascular diseases. Despite numerous reports suggesting a substantial genetic factor contributions to the pathogenesis of type 2 diabetes and diabetic nephropathy, no major susceptibility gene has been identified so far (1, 2). However, several immunogenetic variabilities have been linked to renal disease and type 2 diabetes. For instance, significant variations in major histocompatibility complex had been found to be relevant to renal disease (3). HLA-DR2 and HLA-A3 are associated with membranous nephropathy, while IgA nephropathy is associated with HLA-DR1 and HLA-B35 (4). In addition, polymorphism of several proinflammatory cytokines such as TNF- $\alpha$ , TNF- $\beta$  and other cytokines have been shown to be linked with the severity of many inflammatory disorders in type 2 diabetes (5).

Hemodynamic alterations seen in diabetic patients is mediated by the renin-angiotensin system which plays a role in inflammatory and immune responses leading to activation, proliferation and hypertrophy of glomerular and tubular cells and infiltration of macrophages and lymphocytes (5). Therefore, RAS has a key role in the pathogenesis of cardiovascular and renal complications of diabetes. Activation of glomerular renin-angiotensin system (RAS) in DN patients occurs in more than 80% of patients and seems to be due to elevated glucose level (6). Using ACE inhibitors to reduce the coronary heart disease risk, a decline of 25-30% in progression of impaired glucose tolerance to type 2 diabetes was shown (7). The D allele leads to a higher ACE expression and activity and therefore might predispose individuals to type 2 diabetes (8). Nelson et al. and Chowdhury et al. have shown that DN is an important complication of DM of both type 1 and type 2 (9-10).

Angiotensin-I converting enzyme (ACE) is a Zinc metallopeptidase widely distributed on the surface of endothelial and epithelial cells. By stimulation of renin, angiotensinogen is converted to angiotensin-I. Then, ACE converts Ang-I to Ang-II, a potent vasopressor peptide which is the main active product of the RAS. It is hypothesized that higher ACE activity could result in increased conversion of Ang-I to Ang-II which plays an important role in renal pathology (3-4).

Recent studies demonstrate that Ang-II is a potent pro-inflammatory agent which modulates immune and inflammatory responses such as chemotaxis, proliferation and differentiation of monocytes to macrophages (6). Ang-II induces the adhesion of monocytes and neutrophils to endothelial cells through the production of P-selectin, intercellular adhesion molecule type 1 (ICAM-1), and vascular cell adhesion molecule type 1 (VCAM-1) on vascular endothelial cells and smooth muscle cells in vivo and in vitro (6).

Elevated serum levels of VCAM-1, ICAM-1, IL-6 and TNF- $\alpha$  are seen in hypertensive and diabetic nephropathy (DN) patients (5). In endothelial cells, Ang-II increases IL-1 production and in macrophages up-regulates TNF- $\beta$  gene expression (5). Accordingly, it is obvious that genetic polymorphisms of ACE might influence the progression of diabetic nephropathy and the response to treatment to renoprotective regimens (11-13).

The ACE gene is located on the long arm of chromosome 17(q17). Insertion or deletion of a 287 base pair fragments in the 16<sup>th</sup> intron of the ACE gene may determine its genotype (14-16). Based on the presence or absence of the 287 base pair sequence

in intron 16, three genotypes exist: DD, II homozygotes and ID heterozygote (8, 17). Since the ACE I/D polymorphism determines the total plasma ACE concentration, ACE might be a good candidate gene for type 2 diabetes (18).

## MATERIALS AND METHODS

ACE gene polymorphism was studied in 170 patients (85 type 2 diabetics with nephropathy and 85 type 2 diabetics without nephropathy) and 91 control subjects in Tehran, Iran. Patients were recruited from diabetes clinic of the Imam Hospital, Tehran University of Medical Sciences, Tehran, Iran. Informed consent was obtained from the patients and the control subjects. Controls did not have any abnormalities regarding their physical examination, blood pressure, family history, urine analysis and routine laboratory blood tests and none of them were receiving any medications at the time of participation.

After a 12-hour overnight fasting, 10 ml of 15% EDTA anticoagulated blood sample and 5 ml of blood without anticoagulant were obtained from each patient and the control and centrifuged within 2 hours. The weight of the subjects were recorded and their body mass index ( $BMI = \text{Weight} / \text{Height}^2$  (kg/M<sup>2</sup>)) was calculated. Nephropathy was defined as the presence of macroalbuminuria (>300mg/day).

**DNA Isolation and Determination of ACE Genotypes.** Genomic DNA was isolated from peripheral blood leukocytes according to a standard salting out method (19). For amplification, a flanking primer pair was used and when it was necessary, a primer pair that recognizes the insertion specific sequence was also employed for confirmation of the specificity of the amplification reactions (20-21). PCR was performed with 20 pm of each primer (sense primer: 5'-CTG GAG ACC ACT CCC ATC CTT TCT 3' and anti-sense primer: 5'-GAT GTG GCC ATC ACA TTC GTC AGA T-3') in a final volume of 25  $\mu$ l, containing 0.5 mg genomic DNA, 2 mM MgCl<sub>2</sub>, 10 mM Tris-HCl (pH=8.3), 0.2 mM of each dNTP and 0.5 unit of Taq polymerase. PCR was done with an initial denaturation at 94 °C for 1 min. Then the DNA was amplified for 30 cycles with denaturation at 94 °C for 30 sec, annealing at 58 °C for 30 sec, and extension at 72 °C for 1 min followed by final extension at 72 °C for 8 min.

After electrophoresis in a 2% ethidium bromide-stained agarose gel, the PCR products were visualized under UV light. In case of the deletion (D allele) and insertion (I allele), a 190 bp fragment and a 490 bp fragment were obtained, respectively. Therefore, there will be three genotypes after electrophoresis: A 490 bp band (genotype II), a 190 bp band (genotype DD) and both 490 and 190bp band (ID genotype). Mistyping of ID heterozygote as D homozygotes may occur. Thus, each sample which had the DD genotype was submitted 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 °C. The reaction yielded a 335-bp amplicon only in the presence of an I allele and no product when the samples were homozygous for DD.

**Statistical Analysis.** The SPSS software 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 or without nephropathy using  $\chi^2$ -test. Odds ratio (OR) as estimates of relative risk for disease were calculated and 95% confidence intervals was obtained by SPSS logistic regression. Two-tailed Student's t-test was also used to compare quantitative data. P-values less than 0.05

were considered significant.

## RESULTS

As is shown in table 1, type 2 diabetic patients with and without nephropathy were well-matched for gender, age, body mass index (BMI), duration of diabetes, biochemical parameters and HbA-1c values. The mean age of diabetic subjects without nephropathy ( $59.5 \pm 7.6$  years) was slightly higher than that of the DN group ( $59.2 \pm 8.2$  years), but gender distribution was identical in both groups ( $P=1.000$ ). Patients with nephropathy had significantly higher systolic blood pressure values. The studied groups were compared with respect to clinical findings and biochemical parameters. Serum creatinine, fasting blood glucose (F.B.S) levels and proteinuria (mg/day) increased significantly in patients with nephropathy compared to those without nephropathy ( $p < 0.05$ ). The phenotype characteristics of diabetic and control subjects are also shown in table 1.

The average BMI, blood pressure, total cholesterol, LDL-C, HDL-C, VLDL-C, serum creatinine and triglycerides were significantly higher in the diabetic patients compared to the controls ( $p < 0.001$ ). The average age of the diabetic patients ( $59.4 \pm 8$  year) was higher than that of the control subjects ( $45.4 \pm 8$  year). Allele and genotype frequencies of the ACE gene in studied population are shown in table 2 and figure 1. Both patients and controls were in Hardy-Weinberg equilibrium. The frequency of DD, ID and II genotypes among the studied group was; 30.6%, 55.3% and 14.1% in patients with nephropathy, 20%, 61.2% and 18.8% in patients without nephropathy and 14.3%, 46.2% and 39.6% in control subjects, respectively. Analysis of our results showed that the frequency of DD genotype was significantly higher in DN patients in comparison to control group (30.6% vs. 14.3%,  $P=0.0002$ ). However, the frequency of DD genotype was not significantly different between DN patients and patients without nephropathy (30.6% vs. 20%,  $P=0.25$ ). On the other hand, the frequency of D allele significantly increased in DN patients compared to control group (58.2% vs. 37.3%,  $P=0.0001$ ) whereas the difference between DN patients and patients without nephropathy was not significant (58.2% vs. 50.5%,  $P=0.19$ ).

**Table 1. Clinical and biochemical characteristics of type 2 diabetic patients, diabetic nephropathy and normal control subjects**

Variable	Type 2 diabetic patients n=85	Diabetic nephropathy (DN) n=85	Normal control subjects (control) n=91
Age (years)	59.4±7.7	59.2±8.2	45.3±8.3
Sex (M/F)	43/42	43/42	44/47
Diabetes duration (year)	14.4±3.7	11.7±4.7	-
BMI (kg/m <sup>2</sup> )	28±12.8	26.38±3.9	24.43±3.3
Systolic (mmHg)	134.9±17.6	140.7±26	119.5±15
Diastolic (mmHg)	86.3±11.6	89±10.2	77±6.2
F.B.S (mg/d)	204.6±52.1	226.4±63.3	74.5±7
Cholesterol (mg/d)	207.3±39.4	217.8 ± 45.7	165.7±21.1
triglyceride mg/dl	199.2±92.9	198.7±88.3	122.4±48.9
H.D.L mg/d	43.6±9.5	43±9.4	48.5±10.1
L.D.L mg/dl	123.1.53±35.5	121.9±31.1	88.78±18.4
V.L.D.L mg/dl	37.4±10.9	36.9±13.3	23.8±8.4
Urinary protein-mg/day	6.46±3.72	800.2±393.6	Less than 10
HA1c	8.35±1.9	8.8±1.9	-

Data are presented as means±SD. Comparisons were made using Student's t-test (for continuous variables). Statistically significant; \* $p < 0.05$ .

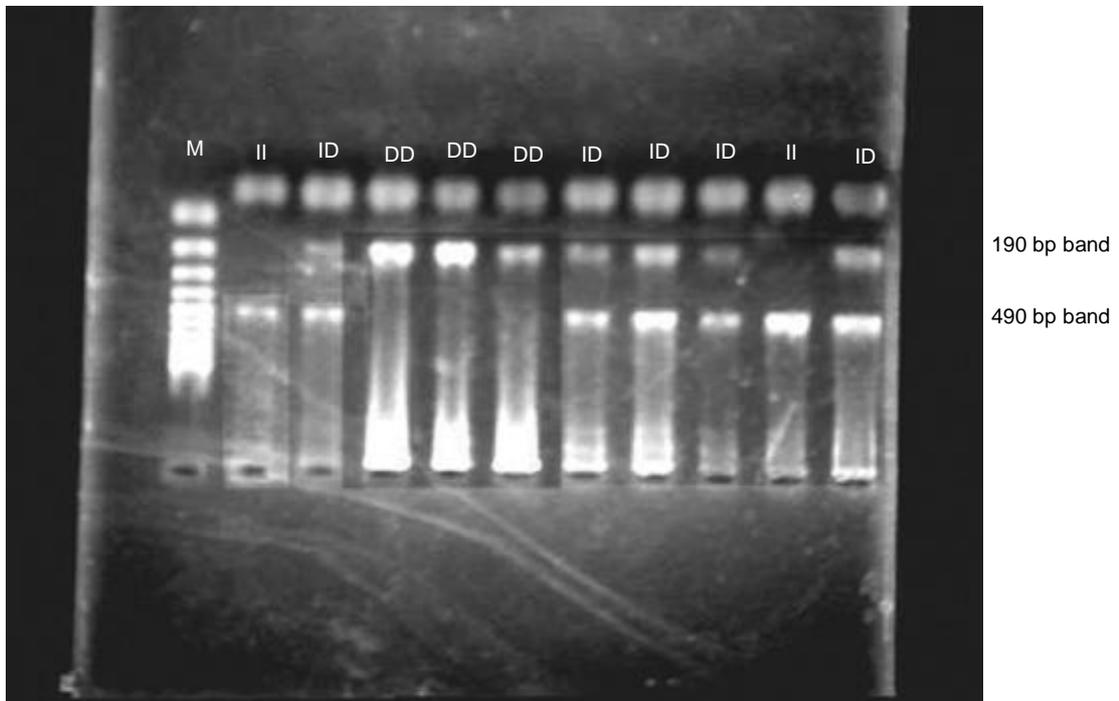
**Table 2. Allele and genotype frequencies of ACE gene insertion/deletion polymorphism in Type 2 diabetes without nephropathy, Type 2 diabetes with nephropathy and control subjects**

Genotypes	DN* n=85		DM** n=85		control subjects n=91		P value Type 2 DM vs. Type 2 diabetes with DN	P value Type 2 DM vs. Control
	n	%	n	%	n	%		
DD	26	30.6	17	20	13	14.3	P=0.25	P=0.0002
ID	47	55.3	52	61.2	42	46.2		
II	12	14.1	16	18.8	36	39.6		
Alleles								
D	99	58.2	86	50.5	68	37.3	P=0.19	P=0.0001
I	71	41.7	84	49.4	114	62.6		

The distribution and comparisons of alleles and genotype frequencies of ACE polymorphism gene in each case was made using Chi-square test, Fisher's exact likelihood ratio.

\*DN;Diabetic nephropathy

\*\*DM;Type 2 diabetes mellitus



**Figure 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

Several immunogenetic markers have been linked to Type 2 diabetes. Ferrannini et al. suggested that the pattern of inflammatory cytokines is important in the pathogenesis of type 2 diabetes. For example, both IL-6 and IL-1 $\beta$  act on liver to produce

the characteristic dyslipidemia of the metabolic syndrome, with an increase in VLDL and a decrease in HDL levels (22).

Joachim et al. evaluated the effects of various inflammatory cytokines on predisposition to type 2 diabetes. They showed that elevated levels of IL-6 and IL-1 $\beta$  had roughly a threefold increased risk for developing type 2 diabetes compared to reference group (12).

Angiotensin converting enzyme (ACE) is a Zinc metallopeptidase which converts Ang-I to Ang-II, a potent vasopressor peptide in renin-angiotensin system (RAS) (23).

Ang-II is a potent pro-inflammatory agent which modulates some immune and inflammatory cell responses, such as chemotaxis, proliferation, and differentiation of monocytes into macrophages. It was hypothesized that the higher ACE activity could result in an increased Ang-I to Ang-II conversion which plays an important role in pathophysiology observed in DN (24).

An insertion or deletion of a 287 base pair fragment in the 16th intron of the ACE gene produces three genotypes; DD, II and ID (19-20). Since the ACE I/D polymorphism is associated with overall plasma ACE concentration, ACE might be a good candidate gene for the type 2 diabetes (1, 2, 6).

In this study, we examined ACE gene polymorphism in type 2 diabetic patients with and without diabetic nephropathy and a control group in Tehran, Iran. Our results showed that the frequency of DD genotype as well as D allele significantly increased in DN patients in comparison to control group while the difference between DN patients and patients without nephropathy was not statistically significant. Our findings are in agreement with some other studies (7, 25, 26).

A meta analysis by Kuns et al. have revealed that the risk of nephropathy increases in the presence of DD or ID genotypes in Asian patients with diabetes but not caucasian patients (27). Jeffers et al. showed a positive association between ACE DD genotypes and diabetic nephropathy (28). However, Schmidt et al. (29) and Liao et al. (30) did not observe any association between ACE genotypes and diabetic nephropathy. This observation emphasizes the importance of geographical and ethnical backgrounds of the subjects participating in the study of ACE genotypes and their association with type 2 diabetes and DN. Other factors such as study design, number of studied subjects, type of diabetes (type 1 vs. type II), and assessment methods in type 2 diabetes may all contribute to the lack of consistency among different studies.

Although the I/D polymorphism is in the intronic region of the ACE gene, many studies showed that the DD genotype is strongly associated with increased serum ACE levels. For instance, Stephen et al. showed that the DD genotype leads to a higher ACE expression and activity and therefore might predispose individuals to type 2 diabetes and its complications (7).

In conclusion, it is suggested that ACE DD genotype and/or D allele may increase the risk of type 2 diabetes in our studied population from Tehran-Iran.

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