

## **Association of the Genotypes of Angiotensin Converting Enzyme with the Type 2 Diabetes Mellitus in Khuzestan Province, Iran**

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### **Abstract**

**Background:** The impact of angiotensin converting enzyme (ACE) gene Insertion/deletion (I/D) polymorphism on etiology of type 2 of diabetes mellitus (T2DM) is well established. Reports show that unlike the II or T2DM genotypes the DD genotype of ACE is correlated with high risk of T2DM. In the present study the relationship between ACE gene polymorphism with the T2DM pathogenesis has been investigated in a population living in Khuzestan Province, Iran.

**Methods:** This case-control study performed on diabetic patients in Ahvaz Province, 99 cases and 100 normal individuals were enrolled in this study. T2DM patients were selected according to the WHO criteria. Blood sample was collected from each patient; DNA was extracted and subjected to PCR amplification using specific primers to identify different ACE genotypes (II, ID and DD).

**Results:** The chi-square analysis showed that lower frequency of DD genotype in patients is associated with increased risk of T2DM (Odds ratio= 0.273, P=0.004). However in patients suffering from T2DM the ID and II genotypes were significantly increased.

**Conclusions:** It appears that in T2DM a decrease in DD genotype of ACE and increases in DI and II genotypes is associated with changes in fasting blood sugar, triglyceride, total cholesterol; and blood pressure. These data may suggest that the DD genotype of ACE is associated with T2DM in Khuzestan Province.

**Keywords:** Angiotensin Converting Enzyme; Polymorphism; Genetics Insertion/Deletion (I/D), Type 2 diabetes mellitus

### **Introduction**

Diabetes is one of the most costly diseases which affects human society both emotionally and financially (Tuei, Maiyoh et al. 2010). Diabetes is a group of metabolic diseases in

which blood sugar of a person is higher than normal range (Ebenibo, Edeoga et al. 2014). American Diabetes Association has categorized diabetes in three main classes. Insulin production in type 1 of diabetes failures and that is why this group is named insulin-dependent type of diabetes (Alqahtani, Khan et al. 2013). The second one, which is the most

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common type, is known as non-insulin dependent diabetes which results from insulin resistance in cells. Gestational type is other class of diabetes that mothers have high blood sugar during pregnancy time with no history of diabetes (Alqahtani, Khan et al. 2013).

High blood sugar in long time makes patients susceptible to a lot of complications and diseases like Alzheimer, Parkinson, Atherosclerosis, retinopathy, nephropathy etc. For example, diabetic nephropathy (DN), causes millions of deaths worldwide, is a chronic disease and one of the major micro-vascular complications of diabetes. In DN, loss of renal function raises urinary albumin excretion and causes abnormal glomerular filtration rate (GFR) (Moresco, Sangoi et al. 2013; Sun, Su et al. 2013). Patients with diabetes encounter with nephropathy in about 20–30% of cases, but in type 1 diabetes larger fraction of these patients progress to end-stage renal disease (Caramori, Parks et al. 2013; Moresco, Sangoi et al. 2013). To control the progression of DN, it is shown that controlling blood pressure and blood carbohydrates beside inhibition of the renin-angiotensin-aldosterone system (RAAS) will be effective (Grothusen, Divchev et al. 2009; Van Buren and Toto 2013). There is a strong relationship between the RAAS and the progression of diabetic renal disease which has been known for decades. The Angiotensin Converting Enzyme (ACE, EC 3.4.15.1) is the key enzyme in renin–

angiotensin–aldosterone system (RAAS), the gene polymorphism of which can affect diabetic nephropathy (Kugaevskaia, 2012; Zhou, Yin et al., 2013; Zhou and Lin, 2015). ACE converts angiotensin I to the potent vasoconstrictor angiotensin II and inactivates a vasodilator known as bradykinin (Ruiz et al. 1994; Koitka and Tikellis, 2008).

The common polymorphism in ACE gene is formed by the insertion (I allele) or deletion (D allele) of a 287–base-pair (bp) DNA sequence within intron 16. The role of this polymorphism has been reported by scientists in micro vascular disorders, especially in diabetes. It has been shown that the carriers of D allele are significantly at higher risk of diabetic nephropathy in comparison with I allele types (Agerholm-Larsen et al., 1997; Conway et al., 2014).

Given the role of ACE gene polymorphism and correlation with diabetic complications in previous studies and also considering high prevalence of DD genotype among diabetic people in different parts of the world, we decided to examine the association between ACE gene polymorphisms and diabetes occurrence in a population living in Ahvaz, South West of Iran.

## **Materials and Methods**

### **Study population**

This case-control study was carried out on 99

patients (male/female; 50/50%) diagnosed as type 2 diabetes (T2DM) which were selected according to the WHO criteria and 100 healthy individuals (male/female; 52/48%).

All the patients were referred to the diabetes clinic of Ahvaz hospitals during 2004-2006. The study protocol was approved by the Medical Ethics Committee of the Ahvaz University of Medical Sciences and it was in accordance with the Helsinki Declaration of 1975 as revised in 1996. Written informed consent was obtained from all participants before collecting blood sample. Blood samples were collected in EDTA (anticoagulant)-containing tubes and stored at 4 °C for further use.

The exclusion criteria was that the patients suffering from cardiovascular disease or other acute and/or chronic diseases. The demographic and clinical characteristics of participants are summarized in Table-1. The cases and controls were age-matched and the age of the both patients and controls was ranging from 25 to 55 years. The patients were on regular anti-diabetic medication i.e., Metformin but received no insulin treatment.

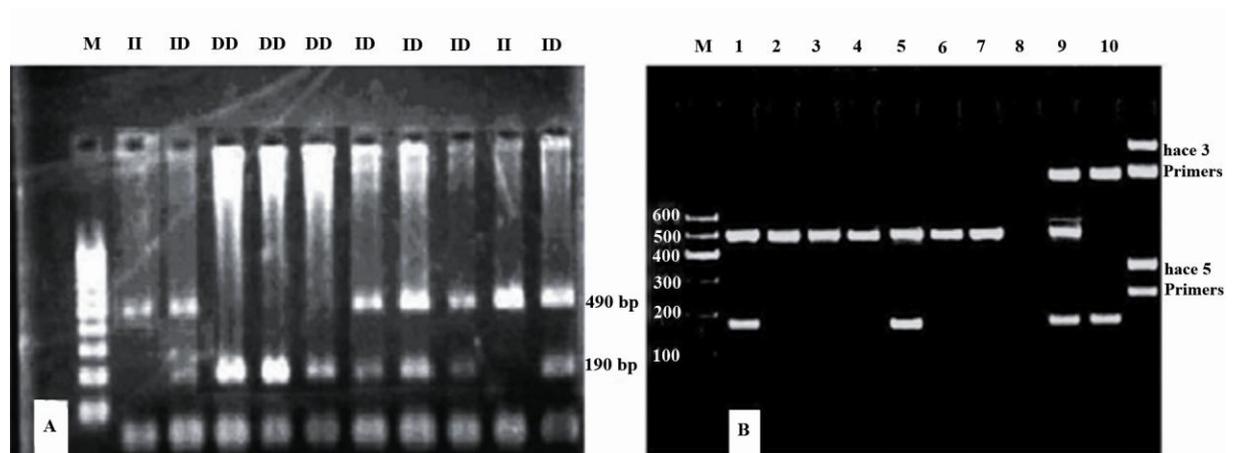
#### **Determination of ACE genotypes**

DNA was extracted from whole blood using DNA saturated salt method according to the Nucleon BACC2 kit (Tepnel Life Sciences, Manchester, UK). The Insertion (I)/Deletion (D) alleles were identified based on PCR

amplification method (Tekken thermo cycler) using specific primers to detect the ACE alleles as amplicons of 490 and 190 bps, respectively. In brief, PCR reaction mixture was prepared using 50 ng of both forward and reverse primers (5' -CGTGAGACCACTCCCATCCTTTCT-3' for forward primer and 5' -GATGTGGCCATC ACATTTCGTCAGAT-3' for reverse), one unit of *pfu* DNA polymerase enzyme (Stratagene, La Jolla, California), 500 μM of dNTP, 100 ng of genomic DNA (template), and 3 mM magnesium chloride. The final volume of the reaction mixture was 50 μl which was adjusted by adding PCR buffer. The following protocol was used to PCR performing; initial denaturation at 95° C for 5 min followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 67°C for 1 min and extension at 72°C for 2 min. The PCR procedure was terminated by a final extension at 72°C for 5 min. PCR products were separated on 2% agarose gels for determination of homozygosity or heterozygosity of D (190 bp) and I (490 bp) alleles (Figure 1). Since the D allele was dominantly amplified in heterozygous samples, each sample identified as DD genotype was again subjected to an independent PCR amplification using a primer pair (5' -TCGGACCACAGCGCCCGCCACTAC-3' for forward primer and 5' -TCGCCAGCCCTC CCATGCCATAA-3'

for reverse) that recognizes insertion-specific sequences. The PCR condition used in this case was similar except for an annealing temperature of 67°C. The PCR amplicon was a

335-bp fragment observed in presence of an 'I'-allele and no product in samples being homozygous for DD (Lindpaintner et al., 1995).



**Figure 1.** Determination of ACE genotypes by PCR amplification. **A.** this figure shows Deletion 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. **B.** this figure indicates screening for the erroneous assignment of the DD genotype to DI samples with use of an insertion specific primer. At the top, several samples (lanes 1 through 6) are identified as DD by the hace3 primer pair, followed by a blank control (lane 8) and three standards for DD, DI, and II (lanes 7, 9, and 10, respectively). The DI standard in lane 9 is an example of preferential amplification of the D band as compared with the I band; a presumed heteroduplex band (faint line below the band) is also present. At the bottom, the same samples were amplified with the hace5 primer pair, which indicates the insertion-specific sequences. A sample previously misclassified as DD appears in lane 5.

### Statistical analysis

All data were analyzed using SPSS version 15. Frequencies of genotypes (DD, II, DI) were compared with the values predicted by assumption of Hardy-Weinberg equilibrium by Chi-square test. Odds ratio was determined for all samples as a measure of association of the ACE genotype with the phenotype of T2DM. Quantitative parameters were compared by student t-test and P-value <0.05

is considered significant.

### Results

#### Characteristics of the study subjects

Table 1 indicates the distribution of other risk factors in control and cases and the differences in the prevalence of recognized risk factors are summarized. As shown in Table 1, some biochemical/physiological parameters such as body mass index (BMI), Triglyceride (TG),

fasting blood sugar (FBS) and systolic blood pressure were significantly higher in diabetic patients as compared to control group. Other

biochemical and hematological parameters in diabetic patients were within the normal range.

**Table1.** Demographic and biochemical characteristics of patients and normal individuals.

	<b>Controls N=100</b>	<b>Diabetics subjects N=99</b>	<b>P-value</b>
<b>Gender(M/F)</b>	50.51/49.49	50/49	NS
<b>Mean age(years)</b>	40.5	44.1	NS
<b>BMI(kg/m<sup>2</sup>)</b>	25.42±0.36	28.65±0.89	P<.01
<b>Total Cholesterol (mg/dl)</b>	187.53±7.53	201.33±6.66	P<.05
<b>TG(mg/dl)</b>	147.46±24.3	227.76±17.79	P<.05
<b>FBS(mg/dl)</b>	106.66±15.43	166.46±13.64	P<.01
<b>Serum Creatinine (µmol/L)</b>	0.95±0.06	1.00±0.04	NS
<b>HDL(mg/dl)</b>	58.40±4.32	59.96±1.92	NS
<b>LDL(mg/dl)</b>	93.03±8.18	95.8±4.50	NS
<b>Systolic Blood Pressure(mmHg)</b>	117.00±1.52	132.50±2.01	P<.001
<b>PLT(10<sup>+3</sup> /µl)</b>	255.06±18.29	261.76±10.55	NS
<b>MPV(fl)</b>	9.49±0.30	9.78±0.16	NS
<b>PDW(fl)</b>	12.46±0.69	12.58±0.28	NS

M/F: male and female, NS: not significant

**Association of genotype and phenotype**

The frequency of genotypes is demonstrated in Table 2. In healthy subjects 12% of the samples had two alleles of I, whereas in diabetics patients this percentage was 20.2. In healthy individuals

The frequency of one D allele in healthy individuals and patients was 44% and 59.5% respectively. As shown in Table 2, individuals with two D alleles were 44 and 20.2 percent in control group and diabetics respectively.

**Table2.** Frequency of Angiotensin Converting Enzyme genotypes in healthy and diabetic subjects.

	<b>Controls</b>	<b>Diabetics subjects N=99</b>	<b>P-value</b>
<b>II Genotype(%)</b>	12	20.2	P<0.05
<b>ID Genotype(%)</b>	44	59.5	P<0.05
<b>DD Genotype(%)</b>	44	20.2	P<0.001

Table 3 indicates the relationship between the ACE genotypes with phenotype (diabetic and non-diabetic). The odd ratio for II/ID is 0.805

(P=0.683) showing that there is no significant difference between II and ID genotypes and phenotypes. However, the II/DD odds ratio

was 0.273 (P=0.004) which was significantly different between the II and ID genotypes and phenotypes. Also, there was a significant

difference between ID and DD genotypes and phenotypes with odds ratio of 0.339 (P=0.001).

**Table3.** Comparison of Angiotensin Converting Enzyme genotypes association with phenotypes (diabetic and non-diabetic).

Genotype	Odds ratio	CI (95%)	P value for Pearson chi square
II/ID	0.805	(0.35 6 - 1.818)	0.683
II/DD	0.273	(0.0112 -0.004)	0.004
ID/DD	0.339	(0.176 - 0.654)	0.001

Odds Ratio, CI (confidence interval), and P value for different genotypes

### Discussion

In this study, the impact of ACE gene polymorphism in etiology of type 2 diabetes mellitus has been investigated. It has been suggested that the deletion polymorphism of ACE are linked to some important complications of diabetes in men such as myocardial infarction(Ruiz et al. 1994; Grothusen et al., 2009;Dayer et al., 2014).

It has been reported that ACE inhibitors have therapeutic application against diabetic complications(Grothusen et al., 2009; Standl et al., 2012). Here we report a significant relationship between DD genotype of ACE and diabetes occurrence in a group of patients living in Ahvaz, Sothern Iran. Earlier a similar finding was reported in a Chinese population suffering from T2DM (Feng et al.,2002). In this study the DD genotype was associated with increased susceptibility to T2DM (Ha 2014). However, others showed that the II genotype is associated with type 2 diabetes

(Raza et al. 2014). This controversy can be partly attributed to ethnic background. Moreover, various protocols for performing experiments and different criteria for selection of patients can be considered as challenging aspects of this area.

The physiological importance of ACE I/D polymorphism is its association with plasma ACE activity. According to previous studies, the presence of DD genotype is associated with increased ACE activity; whereas those with the II genotype had lowest ACE expression (Hsieh et al. 2000;Fathi et al. 2015).The findings of the present study are in favor of the finding that the DD genotype of ACE plays a role in T2DM pathogenesis. In this connection we showed that patients with DD genotype are more susceptible for diabetes than II and ID carriers.

Overall results showed a significant relationship between DD genotype of ACE and occurrence of diabetes as compared to II and

ID genotypes. This was further supported by showing that that reduction in DD genotype of ACE and increase in the DI and II genotypes frequency were associated with markers such FBS, TG, TC, blood pressure. It is therefore suggested that DD genotype of ACE unlike other genotypes, plays a crucial role in the pathogenesis of T2DM in the population studied. Further studies with different populations in different regions to be carried out to better understand the relationship between ACE polymorphisms and occurrence of T2DM pathogenesis.

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