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Angiotensin Converting Enzyme (ACE) Gene Polymorphism And The Risk Of Diabetic Nephropathy In Type 2 Diabetes

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Abstract: One of the major complications of long term type 2 diabetes is nephropathy. An insertion/deletion (I/D) polymorphism of the gene encoding angiotensin-I converting enzyme (ACE) is shown to have association with diabetic nephropathy. The aim of this case control study was to investigate the possible role of ACE gene in the pathogenesis of nephropathy in patients with diabetes mellitus. The study included 196 subjects (145 T2DM and 51 normal control) T2DM were classified into 2 groups: 97 diagnosed with DN, and 48 diabetic without nephropathy as +ve control. randomly selected was conducted to assess the association of SNP ACE gene polymorphism with diabetic nephropathy in Iraqi population. Blood samples from subjects and controls were analyzed to investigate the ACE(I/D) genotypes. The homozygous genotype (DD) was significantly (OR=2.73, CI 95% 1.1- 7.3, P= 0.04) increased the risk of DN two folds with respect to those of the wild type (II) after adjustment for age, sex and BMI. Also significant variation was obtained when the analysis was carried out without adjustment (OR=3.95, CI 95% 1.7- 9.2, P= 0.001). Similarly the ID genotype significantly (OR = 3.68, CI 95%=1.5- 8.7, P= 0.03) raised the risk of DNA by three folds. dominant and recessive models highlighted significant (P= 0.003) association of dominant model with the risk of DN which raised by three folds. the minor allele frequency (D) was significantly higher (P=0.04) in DN when compared with that of the normal control group.

Keywords: DNA, Periodic study, diabetic nephropathy,.

I. Introduction

Diabetes is among the major causes of renal failure. 10-20% of fatalities in diabetic patients are related to renal failure(1). Type II diabetes and diabetic nephropathy are obviously chronic progressive disorders that are related to a group of genetic, lifestyle and environmental factors(2).The exact etiology is unknown, but hyperglycemia and high blood pressure are considered to contribute to diabetic nephropathy. Persistent high blood sugar or blood pressure levels are two factors that can damage the kidneys, making them incapable of filtering wastes and remove water from your body (3). It is a major cause of end-stage renal disease. There are glomerular hemodynamic derangements leading to glomerular hyper-filtration, causing glomerular damage as indexed by microalbuminuria. There is overt proteinuria, decreased glomerular filtration rate, and end-stage renal failure (4). Angiotensin converting enzyme (ACE) polymorphism is a key element of Renin -Angiotensin-Aldosterone System. The major role of ACE is the transformation of Angiotensin I to vasoactive, natriuretic octapeptide angiotensin II and is thus involved in the pathogenesis of diabetic nephropathy(5)

Angiotensin II (Ang II) is an effective vasoconstrictor of the systemic and the local blood pressure (8-12). Ang II increases systemic and glomerular blood pressure, up regulates mesangial cell proliferation and tissue growth (6). The gene encoding ACE is found on chromosome 17q23. This gene encodes both ACE isoforms, but has two distinctive promoters resulting in different mRNAs (7). There is a genetic variability within the gene, a 278base pair insertion/deletion (I/D) polymorphism, resulting in three different ACE genotypes: I/I, I/D and D/D (8). As the polymorphism is found in an intron, it will not change the structure of the enzyme, However, the polymorphism is strongly linked to the level of ACE in plasma, where I/I, I/D and D/D have low, medium and high levels respectively (9,10). Genes encoding elements of the rennin-angiotensin system have been advocated as predisposition determinants for hypertension, and cardiovascular disease, both of which are common in individuals with diabetic nephropathy (11).

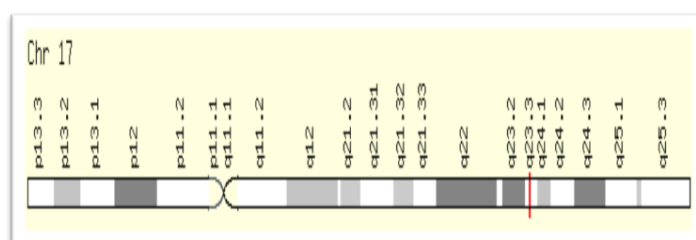


Figure 1: Location of ACE gene in chromosome 17⁽¹²⁾

II. Theory And Experiment

Research Design and Method: A case-control study of 196 subjects (145 T2DM and 51 normal control) T2DM were classified into 2 groups: 97 diagnosed with DN, and 48 diabetic without nephropathy as +ve control. randomly selected was conducted to assess the association of SNP ACE I/D polymorphism gene with diabetic nephropathy in Iraqi population.

Inclusion criteria:

1. Those patients who were diagnosed by physicians as having type 2 diabetes, the criteria to diagnose diabetes were based on WHO guidelines.
2. A subject is said to have diabetes if his/her fasting glucose level was >126 mg/dl (7.0 mmol/l) +symptoms of diabetes (polyuria, nocturia, polyphagia and weight loss).
3. Albuminuria (>300 mg/24 h or >200 µg/min or >200 mg/L) in two of three consecutive measurements on sterile urine samples—with or without renal failure (serum creatinine >150 µmol/L).
4. Albumin/creatinine ratio > 2.5
5. Age of patients was >40 years old.
6. Exclusion criteria:
7. Those diagnosed with T1DM.
8. congestive heart failure.
9. chronic kidney disease.
10. urinary tract infection.
11. Heamaturia.
12. acute febrile illness.
13. Laboratory analysis: Five milliliters of blood were taken from all subjects collected in two tubes, 2.5 ml in plain tube without anticoagulants and 2.5 ml in EDTA tube. After that, the serum tube centrifuged at 2000 xg for approximately 10 minutes, the sera were aspirated and divided into two aliquots, and stored at (-20 C) until time of use. The EDTA tube was used for extraction of DNA.
14. DNA extraction: Peripheral blood samples of patient and control groups were collected in EDTA tubes, and then DNA was extracted from whole-blood samples using the Genomic DNA Mini Kit (Blood / cultured cell) (Geneaid) (13). The ACE gene polymorphism was detected by polymerase chain reaction (PCR) according to the method described by Eleni S et al (14). The template DNA (0.5 µg per sample) was amplified using the following primers: (forward) 5'-AGG CCC TAT GGT AGT GCC TTT-3' and 5'-TCT CTT AGT GCT GTG CTC AC-3' (reverse). These primers (10 pmol of each) were added to a mixture containing 0.2 µmol/L each of dATP, dCTP, dGTP, and dTTP; 5 µL of 10× Cetus buffer (pH 8.3); 5 µL of DMSO (100%); and 0.5 units of Taq DNA Polymerase (Perkin Elmer Cetus), in a final volume of 20 µL.

The PCR was initiated with a denaturation by first heating the samples for 10 min at 94 °C. Thirty cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 58 °C, primer extension for 1 min at 72 °C, and a last extension for 7 min at 72 °C were applied for amplification. The PCR products of the ACE gene locus were examined by gel electrophoresis (2% NuSieve agarose-agarose) at 75 V for 120 min and visualized at room temperature under UV light after ethidium bromide staining. Statistical analysis: Continuous variables expressed as mean ±SD and Student's t-test was used to determine differences in means between control and T2DM groups. ANOVA test and Student's t-test was used to compare mean levels of continuous characteristics across genotypes using SPSS v. 20.0 software (SPSS Inc., Chicago, IL). Categorical data (genotypes and alleles) were expressed as frequency. In all statistical analysis the level of significance was <0.05. Hardy-Weinberg equilibrium (HWE) is a mathematical relationship that related genotypes to allele frequencies (15). Genetic power was calculated using the online software OSSE {online sample size estimator} (osse.bii.a-star.edu.sg⁽¹⁶⁾).

III. Results And Discussion

Age: The diabetic and control groups were found to be matched in age. The distribution of age between the two groups was shown in figure 2.

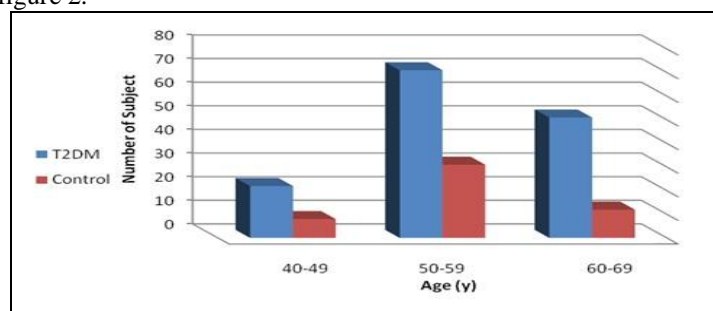


Figure 2: Age distribution of study subjects.

Gender: The diabetic and control groups were found to be matched in gender. The distribution of gender among the two groups was shown in figure 3.

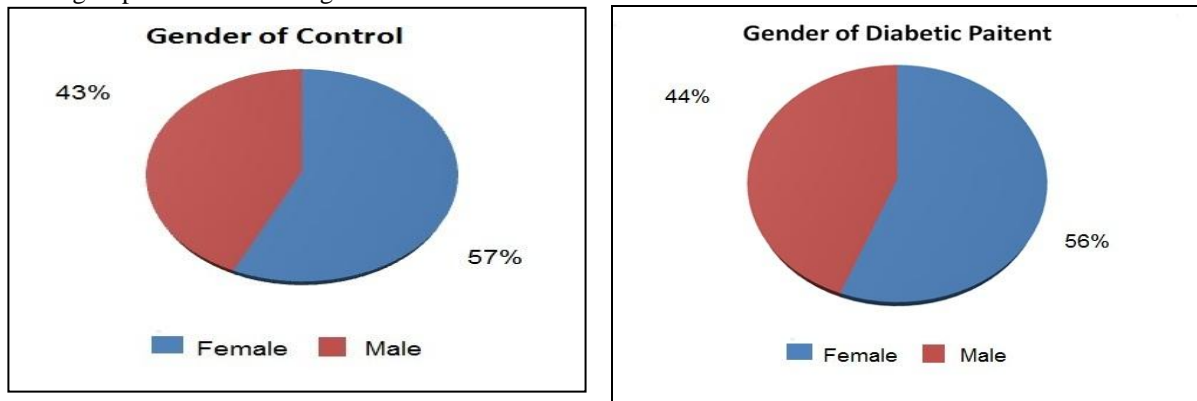


Figure3: Gender distribution of study subjects.

Results of amplification reactions: The amplification product of ACE gene polymorphism as described by Eleni S et. al⁽¹⁴⁾. Results were confirmed by agarose gel electrophoresis (figure 4).

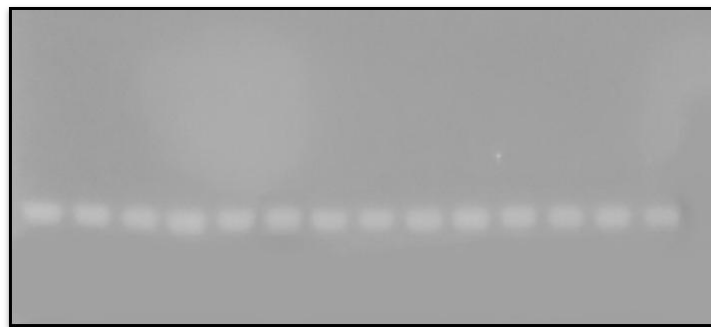


Figure 4: PCR product of DNA analyzed by agarose gel electrophoresis.

DNA was extracted from blood, The PCR product was electrophoresed on 2% agarose (75V and 120 min.) and directly visualized with ethidium bromide under UV light.

The PCR product of ACE gene polymorphism was analyzed by agarose gel electrophoresis. Results demonstrated one (490 bp), one (190 bp) and two (490 , 190 bp) bands for those with wild type (II), homozygous (DD) and heterozygous (ID) genotypes respectively (figure 5).

The overall power of the current study to detect a significant difference in level of 0.05 was estimated and found to be 90.8 % for ACE gene polymorphism. Genotype frequencies of ACE were consistent with Hardy–Weinberg equilibrium in T2DM and control subjects (P< 0.05).

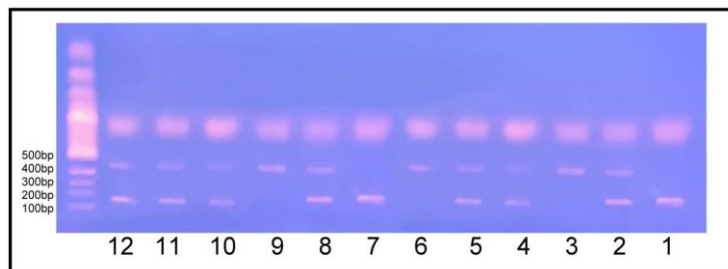


Figure 5: Results of ACE gene polymorphism product on agarose gel electrophoresis. A PCR product of ACE gene was electrophoresed on 2% agarose (75V and 120 min) and directly visualized with ethidium bromide under UV light.

Lines: DNA Marker

Lines 2, 4, 5,8,10,11,12: ID genotype 490,190 bp

Lines 3,6,9 II genotype 490 bp

Lines 1,7 DD genotype 190 bp

Genotype and allele frequencies: To determine the genotyping error rate, a random duplication in 10% of the samples was analyzed. The concordance was obtained to be 100%. The genotype and allele frequencies of ACE gene polymorphism in T2DM with nephropathy and normal control subjects in codominant, dominant and recessive models were examined by multinomial logistic regression analysis.

The homozygous genotype (DD) was significantly (OR=2.73, CI 95% 1.1- 7.3, P= 0.04) increased the risk of DN two folds with respect to those of the wild type (II) after adjustment for age, sex and BMI. Also significant variation was obtained when the analysis was carried out without adjustment (OR=3.95, CI 95% 1.7- 9.2, P= 0.001). Similarly the ID genotype significantly (OR = 3.68, CI 95%=1.5- 8.7, P= 0.03) raised the risk of the DN by three folds. In this sense the odds ratio without adjustment revealed no significant alteration.

Further analysis regarding the dominant and recessive models highlighted significantly (P= 0.003) association of a dominant model with the risk of DN which raised by three folds respectively. However, results of recessive models failed to do so. On the other hand the minor allele frequency (D) was significantly higher (P=0.04) in DNA when compared with that of the normal control group.

The genotype and allele frequencies of ACE gene polymorphism in T2DM with nephropathy and normal control subjects in codominant, dominant and recessive models were examined by multinomial logistic regression analysis (table 2).

The table 2 reveal no significant alteration between DN and diabetic control without nephropathy in the homozygous genotype (DD) and heterozygous(ID) with and without adjustment.

Further analysis regarding the dominant and recessive models highlighted no significant association of dominant model and recessive with the risk of DN. On the other hand the minor allele frequency (D) was not significantly affected in DN when compared with that of the positive control group.

Table 1: Results of genotype and allele frequency of ACE (rs1799752) gene polymorphism in patient and healthy control groups

ACE (I/D)	Control -ve n=51	DN n=97	Unadjusted OR (95% CI)	P value	Adjusted OR (95% CI)	P value
Codominant						
II(Reference)	20	16				
ID	18	56	3.95 (1.7-9.2)	0.001	3.68 (1.5-8.7)	0.03
DD	13	25	2.3 (0.8-5.9)	0.08	2.73 (1.1-7.3)	0.04
Dominant						
DD+ID	31	81	3.2 (1.5-7.1)	0.003	3.5 (1.5-7.9)	0.002
Recessive						
II+ID(Reference)	38	72				
DD	13	25	0.97 (0.4-2.1)	0.94	0.93 (0.4-1.9)	0.8
Frequency of D allele	44 (43.1%)	106 (54.6%)	1.65 (1.1- 2.6)	0.04		

Table 2: Results of genotype and allele frequency of ACE gene polymorphism in patient and +ve control groups

ACE (I/D)	Control +ve n=48	DN n=97	Unadjusted OR (95% CI)	P value	Adjusted OR (95% CI)	P value
Codominant						
II(Reference)	10	16				
ID	28	56	1.27 (0.51- 3.16)	0.6	1.27 (0.4-3.2)	0.6
DD	10	25	1.36 (0.47- 3.95)	0.5	2.87 (0.8-9.6)	0.08
Dominant						
DD+ID	39	81	1.2 (0.5- 3.1)	0.5	1.2 (0.4-3.1)	0.6
Recessive						
II+ID(Reference)	38	72				
DD	10	25	1.2 (0.5- 2.9)	0.5	0.9 (0.9- 1.1)	0.8
Frequency of D allele	48 (50.0%)	106 (54%)	1.3 (0.8- 2.1)	0.2		

Genotypes and allele frequencies of ACE gene: Diabetic nephropathy is now the most common cause of end stage renal disease. Some diabetic patients develop nephropathy, whereas others do not, despite having a long-term hyperglycemia. Because known environmental factors do not fully explain this, researchers have sought the answer at the genetic background of the host.

ACE polymorphism appears to have a significant impact on the progression of diabetic nephropathy. Several studies have found the D allele to be an independent risk factor for diabetic nephropathy and it is used as a marker in population structure analyses^(17,18,19).

To our knowledge, there have been no studies on ACE gene polymorphism in the Iraqi population and in view of the high prevalence of diabetic nephropathy, it is important to look for the gene association in the Iraqi population, and compare it with previous studies on the same issue.

Genotype frequencies of ACE were consistent with Hardy–Weinberg equilibrium (HWE) in control individuals and DN subjects ($P=0.08$). These findings were also reported by Sikdar M et al⁽⁵⁾, Golmohamadi T et al⁽⁶⁾, Haque et al⁽¹⁸⁾ and Marre et al⁽²⁰⁾.

The calculated genetic power to detect a significant difference at level of 0.05 for ACE gene was 90.2%, which is seemed to be greater than the optimal level (80%).

In the current study ID genotype was the most frequent allele, followed by DD and II of total cases of diabetic nephropathy. These results consistent with the Sikdar M et al, in Eastern Indian populations which found the frequency of DD, ID and II in nephropathic patients were 26.7%, 53.3% and 20.0%⁽⁵⁾. Golmohamadi T, et al, in an Iranian cohort found the frequency of DD, ID and II genotypes in patients with nephropathy to be 30.6%, 55.3%, 14.1%, respectively⁽⁶⁾. While in a North Indian diabetic nephropathy population the frequency was 17.0%, 54.2%, 28.8% for DD, ID and II genotypes respectively⁽¹⁸⁾. This difference is attributed mainly to different ethnic groups^(6,17,18,21).

Results of ACE gene polymorphism demonstrated heterozygous genotype (ID) carriers have three folds risk of development of DN when compared with those of the reference type (II) after adjustment for age, sex and BMI, while the risk in DD genotype carriers was two folds.

Dominant model was demonstrated to rise the risk of DN by three folds. Also the D allele associate with DN. Such observations strongly suggested a role of ACE gene polymorphism in the pathogenesis of DN in Iraqi patients. The current findings are consistent with results of South Indian⁽²²⁾, north Indian⁽²³⁾, Japanese subjects⁽²⁴⁾. In contrast, studies from Poland and Germany, and south Indian population did not show any association between the ACE gene polymorphism and nephropathy between individuals with T2DM^(5,6,25,26,27).

The ACE I/D polymorphism is significantly associated with overt nephropathy, the II genotype being a protective against the disease both in type I and type II diabetics. A possible explanation is that changes in blood glucose may affect renal hemodynamic to a different extent in different genotypes. Indeed, hyperglycemia does not appreciably affect the glomerular filtration rate (GFR) in diabetics with the II genotype and low plasma ACE levels, whereas in those with the ID and DD genotype it induces a GFR increase that correlates with ACE plasma levels⁽²⁸⁾. A possible explanation is that vasodilatation of the arterioles afferent to the glomeruli is NO dependent and is amplified by local angiotensin II levels in a dose-dependent manner⁽²⁹⁾. Thus, in patients with the II genotype, decreased angiotensin II bioavailability secondary to reduced ACE activity may limit glucose-induced preglomerular vasodilatation. In carriers of the I allele, this might translate into less severe hyperfiltration, in particular in those with less effective metabolic control who, in the long term, would be to some extent protected from development and progression of nephropathy⁽³⁰⁾.

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