



Determination of I/D genetic variation of the Angiotensin Converting Enzyme (ACE) gene in Iraqi patients with Renal Failure

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Abstract

This study was proposed to investigate genetic polymorphism in angiotensin-converting enzyme (ACE) gene as a risk factor for renal failure disease incidence. Fifty-eight Iraqi patients (37 men and 21 women) with renal failure undergoing dialysis treatment (hemodialysis and peritoneal dialysis) consulting at Al-Sadr dialysis center were enlisted into this study; the mean age of these patients was (54.25 ± 11.35) years as the first group. A second group was fifty two healthy control subjects collected from donor blood bank (33 men and 18 women) with the mean age of (50.95 ± 12.8) years. The collection of blood samples from renal failure patients and healthy control persons takes place for detection of serum Creatinine, Urea and Angiotensin Converting Enzyme (ACE) as biochemical diagnostic markers. Then, DNA was extracted from the blood of two groups for detection of Insertion/Deletion (I/D) polymorphism in ACE gene in intron 16 as a prognostic marker for detection of renal failure disease.

The result of this study appears that the levels of Creatinine, Urea and ACE enzyme in renal failure patients increased significantly when compared with control. On the other hand, the genetic polymorphism I/D in ACE gene concluded that the DD and II genotypes differences were highly significant between patients and control ($P = 0.021$) especially in patients with DD genotype OR = 3.49 (95% CI = 1.56-7.93). Also, The DD v/s ID+II and DD+ID v/s II genotypes comparison between patients and control group showed a significantly different ($P = 0.001$) with OR=5.874 (95% CI = 3.71-8.80), OR= 3.58 (95% CI = 2.01-6.81) respectively which revealed that the patients with DD genotype have a high risk for renal failure occurrence. In addition, it was detected that serum ACE level was higher in patients with DD genotype in ACE gene than in patients with II+ DI genotypes ($p = 0.015$). The present study concluded that there is a higher prevalence of (DD) polymorphism genotype in ACE gene in renal failure patients which may be responsible for renal failure pathogenesis.

Introduction

Chronic Kidney Disease (CKD) or renal failure is a global prevalent health problem regarded as polyphonic and complex disorder results from interactions between gene-gene and gene-environmental. The pathogenesis of this disease is extremely correlated with genetic variability [1]. The Renin-Angiotensin System (RAS) is an important regulator of kidney function and blood pressure. The correlations between RAS and CKD still unclear on contrary of contributions between RAS and hypertension which is well documented. However, several types of RAS blockers such as angiotensin receptor blockers and angiotensin converting enzyme (ACE) inhibitors have been advising to patients with CDK to prevent renal damage [2]. The individuals differ in their response to treatment with RAS inhibitors depend on pathophysiological characteristics. Thus, several polymorphisms in RAS components are the main factors to contribute to its heterogeneous association between renal failure patients [3]. Angiotensin converting enzyme (ACE) is a main component of the Renin-angiotensin system and has a crucial function in hydrolyzing angiotensin I into a potent vasopressor called angiotensin II that causes regulation of blood pressure, this enzyme has other important functions like degrading a strong vasodilator (Bradykinin), at the same time, this enzyme able to inactivate these two



vasodilators (Bradykinin and angiotensin II) and it has been a role in hydrolyzing Angiotensin and inducing synthesis of the aldosterone-stimulating peptide which causes electrolyte balance [4].

The ACE gene is contained 25 introns and 26 exons and the length of this gene is 21 kilo base, As well the location of the gene on the long arm of chromosome 17. ACE gene has more than 160 gene polymorphisms; the vast majority of them are single nucleotide polymorphisms (SNP). The SNP in this gene involves either a deletion (D) allele or an insertion (I) allele that forms three probable genotypes: DD, II or ID [5]. The deletion (D) or insertion (I) of a 287 base pair Alu repetitive sequence appears in intron 16, this polymorphism is seemed to be related to the interpersonal variability of ACE enzyme levels in circulating blood, the increasing activity of the ACE enzyme in plasma is correlated with the deletion allele at this gene which causes elevation of angiotensin II level that stimulates the expression of other growth factors, leading to kidney problems like glomerulosclerosis [6]. Numerous studies have been done to evaluate the relationship between the progress of renal failure and ACE I/D polymorphism (3, 5, 7). In recent years, there has been a marked increase in kidney disease in Iraq, and for now, the reasons for this are unknown. So, this study focused on the genetic side for this disease as an important aspect of the disease by evaluating the role of ACE I/D in renal failure patients and healthy subjects as a prognostic marker for early detection of this disease. Then, detect the contribution of this polymorphism to serum ACE level.

Subjects & Methods

Subjects

This study conducted through the period from September 2016 to March 2017. The collection of samples was performed in Specialist Centre for Nephrology (Al-Sadr dialysis center) in Al-Sadr Teaching Hospital in Al-Najaf province while the molecular study performed in the laboratory of molecular biology, Faculty of Veterinary Medicine, University of Kufa.

The renal failure patients were (n = 58; 37 men and 21 women), with a mean age (54.25±11.35) years. All the patients were undergoing dialysis treatment (haemodialysis and peritoneal dialysis) following diagnosis of renal failure by nephrologists and taken from Specialist Centre for Nephrology (Al-Sadr dialysis center) in Al-Najaf Province. Diabetic nephropathy patients were excluded from this study; the inclusion criteria for these patients involved a continually increased Creatinine level more than the normal range (from 3.5 to 15.5). Some information was collected for each of the renal failure patients which included gender, age, creatinine, protein uria level. The control group collected from donor blood bank (n=52; 33 men and 18 women) with mean age (50.95 ± 12.8) years, the control subjects had normal kidney functions and they were free from any Kidney diseases, also they were free from any other diseases.

Methods

The collection of venous blood samples were occurred in the morning after the overnight fasting before the dialysis session on a midweek dialysis day. (4 ml) of peripheral blood samples were collected and divided into two groups, the first group includes (1 ml) of blood in ethylenediaminetetraacetic acid (EDTA) tubes which considered as an anticoagulant used for DNA extraction then, the polymerase chain reaction (PCR) technique on these DNA samples was applied for detection of ACE genotype I/D polymorphisms and second group includes (3 ml) in tubes without EDTA allowed to clot for 10-15 minutes, centrifuged and the separated serum is used to analyze the biochemical parameters (Creatinine, Urea and ACE) using Mini vidas technique in renal failure patients as well as in the control subjects.



Determination of ACE Genotypes

Genomic DNA purification kit, (Promega) used for DNA extraction from the blood samples depending on the protocol of Isolating Genomic DNA from whole blood. 100 μ l of the extracted DNA solution were stored at -20 °C. The specific segment of ACE gene was amplified for detection of (I/D) ACE polymorphism by PCR using the specific primers:

Forward: 5' CTGGAGACCACTCCCATCCTTCT 3' ;

Reverse: 3' GATGTGGCCATCACATTCGTCACGAT 5' [8]

PCR amplification was conducted in a total volume of 25 μ l including: 5 μ l DNA (concentration 20 ng), 2.5 μ l of ACE forward primer, 2.5 μ l of ACE reverse primer, 12.5 μ l of 2X Taq green master mix and 2.5 μ l of D. W. PCR tube were closed and transferred into the thermal-cycler when reach temperature reach 95°C and start the amplification program that includes, the reaction were performed in: 4 minute of initial denaturation at 94°C, 32 cycles of 30 second at 94°C, 30 second at 57°C and 1 minute at 72°C and one cycle of 10 minute at 72°C as a final extension. Analysis of PCR results is based on the presence of specific bands of DD, ID and II alleles. The DD homozygous genotype diagnosed by the presence of a single 190 bp PCR product. The II homozygous genotype were distinguished by detecting a single 490 bp PCR product while the ID heterozygous individuals were distinguished by detecting both 190bp and 490bp PCR products as shown in gel electrophoresis [9].

Statistical analysis

All the statistical calculation of the molecular and biochemical parameters were done using statistical software SPSS (version 18). The values are expressed as mean \pm SD for each variable. One-Way Analysis of Variance (ANOVA) used for evaluation of data followed by a test of Tukey's multiple comparisons. Odd ratio with 95 % confidence interval test used for estimation of the gene polymorphism risk. P value < 0.05 was considered a significant.

Results:

Biochemical parameters in renal failure patients and control:

The renal functional parameters were investigated in the current study. The Creatinine level was highly significant increased (P=0.001) in patients group (13.12 \pm 4.98) mg/dL when compared with control (0.71 \pm 0.25) mg/dL. In addition, the level of blood Urea was increased significantly (P= 0.001) in patients (62.22 \pm 10.91) mg/dL than healthy control group (9.65 \pm 2.73) mg/dL. On the other hand, the serum ACE level was seen to be higher significantly (P= 0.01) in patients with renal failure (75.87 \pm 30.11) IU/l than control (28.12 \pm 7.74) IU/l as shown in table 1.

Table1. Biochemical characteristics of renal failure patients and control:

Parameters value	Renal failure patients (Mean \pm SD)	Control subjects (Mean \pm SD)	P-value
Creatinine (mg/dL)	12.91 \pm 4.98	0.71 \pm 0.25	0.001
Urea (mg/dL)	62.22 \pm 10.91	9.65 \pm 2.73	0.001
ACE activity IU/l	75.87 \pm 30.11	28.12 \pm 7.74	0.01

SD: Standard deviation , P: Probability



Distributions of I/D ACE polymorphism in renal failure patients and control:

The percentage of DD, II and ID genotypes in patients group (n = 58) was 18 (31.03%), 21 (36.84%) and 19 (32.75%) patients respectively, whereas among the control group (n = 52) was 4 (7.6%), 35 (67.30%) and 13 (23.07%) respectively as observed in (table 2), (figure 1). The difference of D and I allele genotypes in ACE gene was highly significant between patients and control (P = 0.021). This intensely showed that DD genotype patients have a high chance of renal failure progressing OR = 3.49 (95%CI = 1.56-7.93). Moreover, the pooling of ID with II genotypes v/s DD genotype showed the genotypes comparison were significantly different between patients and control (P = 0.001) with high-risk in patients with DD genotype OR 5.874 (95%CI = 3.71- 8.80). Also, when pooling ID with DD genotypes v/s II genotype, the (P=0.001) and OR continued high OR= 3.58 (95%CI = 2.01-6.81). So, the difference between D and I allele between two groups was significant (P =0.021) with high OR =3.49 (95%CI 1.56-7.93) as shown in table 2.

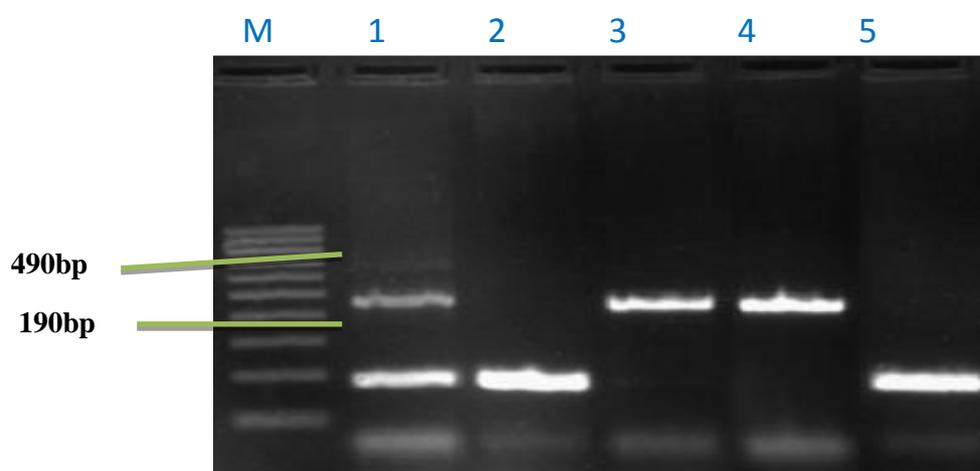


Figure 1: PCR products of ACE gene I/D polymorphism. Lane (M) is 100 bp DNA Ladder. (A): The DD genotype (190 bp, Lanes 2 and 5), the ID genotype (490 bp, 190 bp, Lane 1), the II genotype (490 bp, Lane 3 and 4).

Table 2: Distribution of ACE I/D genotypes among renal failure patients and controls

		Patients (n=58)	Control (n=52)
Genotypes	DD	18(31.03%)	4 (7.6%)
	II	21(36.84%)	35(67.30%)
	ID	19(32.75%)	13(23.07%)
Comparison of different ACE gene polymorphism			
		P-value	OR(95% CI)
	D v/s I	=0.021	3.49(1.56-7.93)
	DD v/s ID+II	=0.001	5.87(3.71-8.80)
	DD+ID v/s II	=0.001	3.58(2.01-6.81)



4-3 Correlation between serum ACE level with (I/D) ACE polymorphism:

Remarkably, the level of ACE enzyme in serum was well associated with I/D polymorphism in ACE, with a prominent effect of the DD alleles. ACE level was observed to be higher in patients with DD alleles than in patients with the II+ DI alleles ($p=0.015$) as illustrated in table 3.

Table 3: Correlation between serum ACE level with (I/D) ACE polymorphism

	DD (n=18)	II+ID (n=40)	P-value
ACE activity (IU/I)	79.11±25.07	41.23±20.91	0.015

Discussion

Progression of renal disease resulted from the interplay of multiple genetic and environmental factors, so, it regarded as a multifactorial disease. Genetic variations can be utilized as a marker to distinguish the people at risk level. Numerous studies have been achieved to distinguish genetic risk causes that stimulate different diseases [10]. The ACE has a vital role in both renal and cardiovascular pathophysiology. The ACE gene variations occur in intron 16. This variation is divided into three kinds: insertion homozygotes II, deletion homozygotes DD and heterozygotes ID [11].

The result of this study revealed that the difference in DD and II genotypes in ACE gene was significant between renal failure patients and control with a high Odd ratio (Risk ratio) in patients with DD genotype. So, the frequencies of DD genotype were significantly increased in patients than control when compared with other genotypes. This result is agreement with several studies like the result of Ali *et al.* that concluded that the repetition of the D allele was higher (42.40%) in end-stage renal disease (ESRD) patients than healthy control persons (31.05%) in the Malaysian population [12]. Other study conducted on Egyptian population showed that the frequencies of DD genotype were increased significantly, while incidences of ID genotype were decreased significantly in the ESRD patients than control with high Odd ratio in patients with DD genotype in ACE gene. So, in the Egyptian population, ACE DD genotype considered as a risk factor for ESRD occurrence [13].

In another studies conducted on Asian population by Tripathi *et al.* [3], Palomo-Pinon *et al.* [14] and Nagamani *et al.* [15], they observed that the DD genotype in ACE gene is the risk factor for the progress of renal failure in Asian people with this disease than healthy individuals with highly significant differences and high Odd ratio in patients with this genotype. Also, The result of this study was accordance to other study demonstrated in Asian and Caucasian individuals [16]. In contrast to those studies, there was a study by Pereira *et al.* failed to confirm the hypothesis that I/D gene variation in ACE has a considerable role in the renal failure risk [17]. Also, the study by Beige *et al.* recorded that there was no correlation between DD genotype in ACE and ESRD, nephropathy respectively [18].

The current study showed that the level of ACE enzyme in serum was increased significantly in renal failure patients than control. This result accordance with the result of Settin *et al.* who documented that the physiological value of I/D variation in the ACE gene is correlated with the activity of ACE enzyme in serum [19]. The DD polymorphism in a person is related with two-fold elevated ACE activity in serum, while the ACE enzyme expression in serum lowest in individuals with II genotype. Furthermore, Elshamaa *et al.* showed a high level of ACE in patients with chronic kidney disease who have DD genotype in ACE gene [20].



The mechanism valuing for the increase of serum ACE level in DD homozygote individuals is unknown. Because the ACE polymorphism is existing in an intron, it may be attributed to disequilibrium with a functional variant of the ACE gene or suppress transcription of ACE gene [21]. DD genotype has been revealed to be associated with the elevation of serum ACE level because elevated ACE protein expression, as a result, the plasma angiotensin II level is increased that stimulates podocyte injury and loss of it which considered as a hallmark of advancing kidney disease [22].

The RAS has been linked with different types of renal diseases. Individuals have DD alleles of ACE gene show increased serum ACE level, therefore may have higher RAS activation. So, carriers of D allele are exposed to a higher level of angiotensin II than carriers of other alleles. As known, angiotensin II participates directly in accelerating kidney damage by supporting cell growth, fibrosis, and inflammation. Hence, when a person has D allele in its ACE gene, more rapid deterioration in renal function is determined than in its absence [23]. Changes in RAS activity is correlated with aging. The participation of molecular and cellular factors and consequent aggregation extracellular matrix compounds with the upgrowth of kidney fibrosis are tightly associated with the renal damage progression as a result of aging [24]. In addition, elevation of ACE activity causes increasing of Angiotensin II levels that enhance growth factors expression and proliferation of mesangial cells and matrix leading to glomerulosclerosis [25]. D allele in the homozygous state of the ACE gene might accord high risk of renal diseases progress. Also, D allele in heterozygous ID state has a high risk of renal failures because of intermediate levels of ACE production in this state. Consequently, the DD genotype and D allele in ACE was correlated with renal ESRD [26].

Obviously, ACE polymorphism is correlated with renal and also cardiovascular diseases; this polymorphism changed the activity of serum ACE and supposed to occur via the pro-inflammatory mechanism. DD genotype Individuals have a twice of serum and tissue ACE levels as individuals with II genotype while ID individuals have intermediate levels. The harmful effect of the deletion polymorphism in this gene on kidney may be an explained by increasing of intra-renal angiotensin II formation and/or insufficient ACE in DD genotype individuals. Consequently, the absence or presence of a 287-bp repeat sequence at intron 16 regarded as a prevalent marker in susceptibility to renal failure occurrence [27].

The present study exposed that the II genotype was increased in healthy controls individuals than in renal failure patients. The lower level of ACE enzyme in this genotype promotes the reduction in glomerular pressure, tubular damage and scarring, and proteinuria that causes a delay of disease progression to a renal failure at the end stage. Another mechanism which interpreted less end stage renal failure outcome in patients with II genotype may be in the control of TGF- β level [28].

Conclusions:

The present study concluded that the meeting of the DD alleles in the ACE gene in the same renal failure patients may lead to this disease, while the assembly of II alleles in the same person may save him against the disease, this revealed ACE gene has greater role in genetic variation as well as prognostic marker for detection of CKD. So, the molecular study could be considered as a reliable technique for early detection and prediction of this disease because it depends on gene level.

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