ACE Polymorphisms Rs (4343) and Its Association with Ace Enzyme in Some of Patients with Diabetic Nephropathy in Some Population of Salah Al-Din Governorate in Iraq

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ABSTRACT
The study was designed to investigate the relationship of *ACE* gene and serum ACE level in a sample of patients with diabetic nephropathy .This study was conducted on (110) blood samples of people with diabetic nephropathy, with (42) samples from healthy people as a control group. The results showed rise in serum ACE level, as it reached (114.59 ± 1.88 U/L) in patients and molecular study showed, after DNA extraction and the use of Real-Time PCR technology for the(ACE) rs4343 gene in patients with diabetic nephropathy and healthy people showed the presence of alleles (A, G) with the appearance of three genotypes, which are (AA, AG, GG). The frequency of the AA genotype is (0.236) the AG genotype is (0.3) and the GG genotype is (0.463) and the value was (OR = 0.66, 95% CI = 0.33 – 1.47) in patients with diabetic nephropathy. The frequencies of the two alleles, A and G, were as follows: for the G allele it was (0.65). and allele A is (0.35). and the(OR = 1.053 , 95% CI = 0.84 – 1.21)

INTRODUCTION

diabetic nephropathy (DN) it is the most common cause of kidney disease in ESRD (end-stage renal disease) and one of the complications of chronic microvascular refractory diabetes (DM). The main pathologic features of DN are tubular atrophy. and tubular interstitial fibrosis[1,2] . In recent years, as a result of the development of genetic detection methods and many studies related to candidate genes that are believed to be related to the disease and after conducting genome-wide association studies some single nucleotide polymorphisms (SNPs) that have an effect on DN have been identified[3]. the great variation in the prevalence and incidence of DN is important in showing that this polygenic disease has a significant impact on the development of DN and that some known genes that cause some cardiovascular diseases, familial hypertension, hyperlipidemia and problems in regulating blood pressure, and some previous studies of these genes showed that some gene polymorphisms such as polymorphism in the ACE gene that is associated with DN as well as in the eNOS gene that is likely to cause DN[4,5]. Among the possible causative factors associated with the development of DN in patients with type 2 diabetes that should be noted are some genetic factors, especially genetic polymorphism in the *ACE* gene[6]. The polymorphism of the *ACE* gene is a key component of the RAAS Renin–Angiotensin Aldosterone System. The main role of ACE is to convert angiotensin I to vasoactive and diuretic angiotensin II, and also contributes to its overall pathogenicity in DN[7]. Several studies have suggested that ACE polymorphisms and a patient's genotype may influence the development, severity, and complications of diabetes[8,9]. It has been shown that the gene *ACE* that encodes the ACE enzyme in humans is located on chromosome 17 q23 and has a variation such as rs464994, rs8066114, rs4461142 and this is a genetic variation within the ACE gene. There is polymorphism in this gene as a result of the insertion / deletion mutation (I / D) 278 bp base pair in intron 16 which leads to three different genotypes of the ACE gene: I/I, I/D, and D/D.[10]

Method

This study was conducted on(152) was (110) blood samples of people with diabetic nephropathy, with (42) samples from healthy people as a control group, collected from the artificial kidney unit in Balad General Hospital and Tikrit General Hospital with ages ranging from 40-70 years. Collected 4 ml of each individual's blood was placed in a tube containing EDTA for the purpose of DNA extraction and use in molecular tests and Collected serum for ELISA.

Table (3-4) Components of the DNA extraction kit

Quick-DNA™ Blood MiniPrep kit	D3025(200 preps)
Genomic Lysis Buffer*	2 x 100 ml
DNA Pre-Wash Buffer**	50 ml
g-DNA Wash Buffer	100 ml
DNA Elution Buffer	2 x 10 ml
Zymo-Spin™ IICR Columns	200
Collection Tubes	400
Instruction Manual	1

Quantitative detection of human ACE concentration in serum

Using the Sandwich ELISA assay, the accurate quantitative detection of human ACE proteins in blood serum

DNA Extraction A blood sample(4 ml) for each individual with diabetic nephropathy and healthy individuals is used to extract DNA in the laboratory by means of the prepared DNA extraction kit consisting of the materials shown in Table (3-4).

Estimation of DNA concentration and purity

A Nano Spectrophotometer was used to estimate the concentration and purity of DNA, where 2 µL of DNA extracted from each sample was used for this purpose and according to the manufacturer's instructions, an estimate of DNA purity was obtained by reading the optical density (OD) at 260 and 280 nm by dividing the first value of the optical density by the second value of the optical density. The acceptable percentage for DNA purity ranges between (1.8-2.0) while its low indicates contamination of DNA with proteins and RNA.

Real Time Polymerase Chain reaction (RT-PCR)

The real-time polymerase chain reaction technique was used to detect the two gene ACE and alleles in samples of (110) patients with diabetic nephropathy and (42) healthy subjects. This important technique is used in many areas of molecular biology research. The name of this technique is abbreviated as RT-PCR and it is also called real time quantitative polymerase chain reaction (q-PCR) [11]. In this research the specialized probe method was used It was named TaqMan method .which contains nucleotide units marked with radioactive particles which allows monitoring after the pairing process between the probe and its complementary nucleic acid. This probe is a dye designed to increase the specificity of the reaction. q-PCR This method was first demonstrated by researcher Kary Mullis in 1991[12].

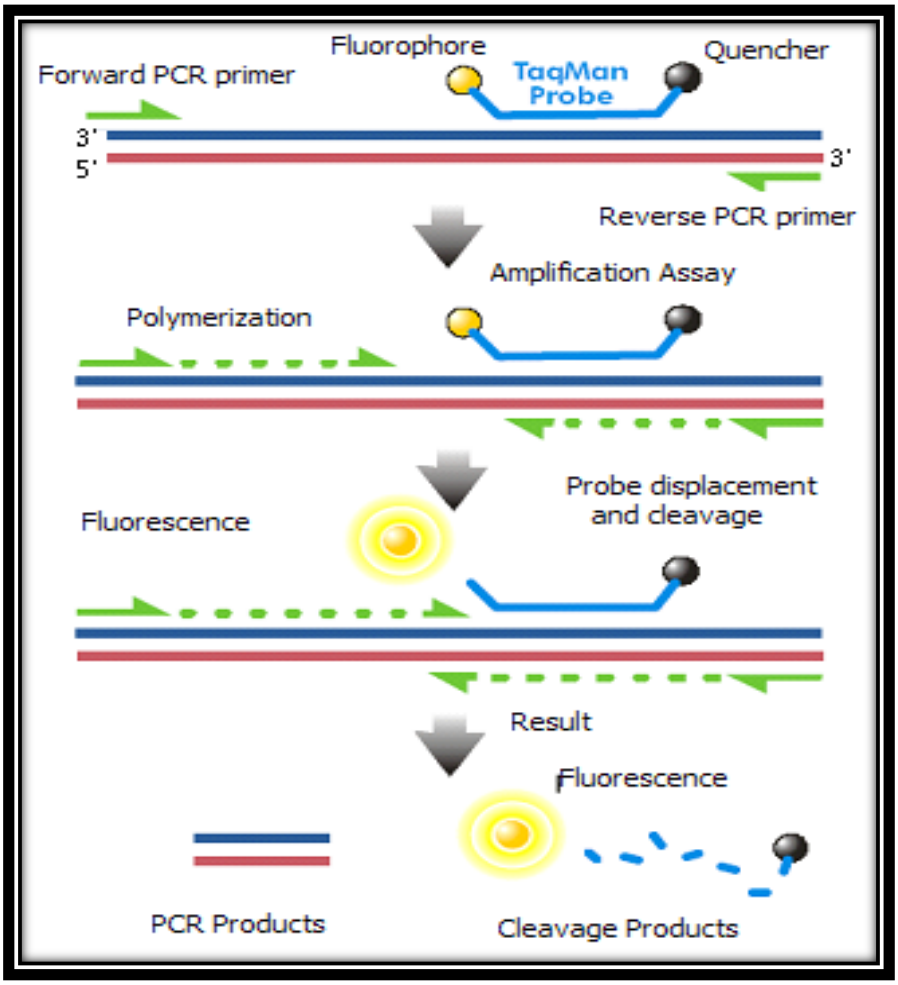

Figure (3-3) shows the installation and working mechanism of the TaqMan probe.

In this study, the ready-made TaqMan probe method was used to detect polymorphisms of the ACE gene loci rs (4343) as shown in Table (3-5)

Table (3-5) showing the location of SNP for the ACE gene.

SNP	Catalog	Assay ID	Sequence
rs (4343)	4351379	C.11942562.20	CAGATCTGACGAATGTGATGGCCAC[A/G]T CCCGGAAATATGAAGACCTGTTAT

RESULTS

1-Results of ELISA for ACE and (DODM)(FHODM) and Biochemical parameters in diabetic nephropathy and healthy.

The results of measuring the level of enzyme (ACE) in blood serum using the ELISA technique for both patients with diabetic nephropathy and healthy people showed a rise in level, as it reached (114.59 ± 1.88 U/L) in patients and Its levels were (U/L 87.47 ±15.05) in healthy subjects. The average of duration of diabetes mellitus (DODM) period between the onset of diabetes and the development of the disease to develop diabetic nephropathy ranged between (11.35 ±4.06 years) in the group of people with diabetic nephropathy and the length of this period plays a role in the incidence of diabetic nephropathy .The results of the same table also showed that the family history of diabetes mellitus (FHODM) is related to the incidence of diabetic kidney failure, as the number of people with diabetes reached (104) samples out of the total number of people with diabetic nephropathy of (110) 94.5% one of their family members had diabetes. The results also showed that there was a rise in the level of glucose in the blood among patients with diabetic nephropathy when compared with the healthy group as it reached (205.30 ± 5.34 mg/dl) in the patients while it reached (120.76 ± 2.96 mg/dl) in the healthy people. The results were measured for urea and creatinine (124.05 ± 3.67 mg/dl) , (3.95 ± 0.12 mg/dl) respectively in the patients.

Table (4-5) shows the results.

Parameters	**The group**	***p*-value**	
	Control Mean ± SE -42	**Patient Mean ± SE -110**	
ACE (U/L)	87.47 ±15.05 b	114.59 ±1.88 a	0.0073*
DODM	_	11. 35 ±4.06	0.03
FHODM	_	(104)(110)	0.08*
Systolic blood pressure (mmHg)	147.84 ± 18.63	155.27 ± 17.92	<0.001
Diastolic blood pressure (mmHg)	84.03 ± 11.41	84. 77 ± 11.62	0.35
Glucose (mg/dl)	120.76 ± 2.96 b	205.30 ± 5.34 a	0.001*
Urea (mg/dl)	34.14 ± 1.59 b	124.05 ± 3.67 a	0.001*
Creatinine(mg/dl)	1.12 ± 0.04 b	3.95 ± 0.12 a	0.001*

The results obtained in the study showed an increase in the level of enzyme (ACE) for the group of patients with diabetic kidney failure when compared to the healthy group. This enzyme is secreted when pressure drops as a result of a decrease in the level of salts in the blood, especially sodium, and this may be due to diabetes or other diseases such as hepatitis, nephrotic syndrome, adrenal insufficiency, hyperthyroidism [13] .The rise in the level of ACE enzyme may be due to the genetic polymorphism of the ACE gene as a result of a mutation in the gene that encodes it and thus leads to an increase in its level in patients with diabetic kidney failure [14]. In a study conducted by[15] on patients with renal failure, it was found that there was a significant increase in the level of the enzyme (ACE) and that this increase is related to polymorphism of the ACE gene. The length of (DODM) has a major role in the development of diabetes towards diabetic nephropathy. The results showed that 67% of patients who had diabetic nephropathy had diabetes during a period of more than 7 years and 33% of them had diabetes for a shorter period. From 7 years as the longer the period of diabetes it leads to an imbalance in kidney function as a result of damage to the small blood vessels in the glomerulus [16]. The incidence of type 2 diabetes (T2DM) at an early age puts individuals at increased risk for premature death especially when complications of diabetes occur[17]. Long-term hyperglycemia and duration of diabetes are known risks for the development of microvascular complications in patients with type 2 diabetes[18]. Concerning (FHODM) that if the mother, father, sister, or brother has diabetes, it is more likely that the individual in the family will develop diabetes[19] .also

considered that family history of diabetes is an indicator of genetic predisposition to diabetic nephropathy due to the involvement of multiple genes and environmental risk factors in the development of diabetes to diabetic nephropathy [20].The results of the biochemical tests showed that the systolic blood pressure was higher in the group with diabetic nephropathy than it was in the healthy group. High systolic blood pressure is also affected by health awareness. Educated individuals who live a healthier lifestyle and avoid an unhealthy diet are less likely to have high blood pressure compared to uneducated individuals[21]. That some behaviors practiced by humans are risk factors in controlling blood pressure it has been shown that lack of physical activity smoking not eating fruits and vegetables consuming foodstuffs that contain a high percentage of table salt and alcohol consumption are significantly related to controlling high blood pressure [22]. In addition there is a significant association between high blood pressure and body mass high blood sugar and hyperlipidemia[23]. The cause may be genetic through a genetic mutation in the genes responsible for the RAAS system, especially the ACE genes AGTR1, AGT It has been proven[24]. that the genes of the RAAS system have an essential role in high blood pressure and thus may lead to diabetic nephropathy (DN). High glucose in the blood is due to many reasons including age as the older a person is the higher the natural rate of glucose in the blood [25]. The level of glucose in the blood is also affected by sex in females as age progresses the level of in glucose the blood rises above the normal rate as a result of its influence on sex hormones it is interesting that hyperinsulinemia or insulin resistance in females especially after menopause for type II diabetics [26]. The reason for the high level of urea in the blood is attributed to many factors including eating meals rich in protein or as a result of reducing the rate of urea excretion from the body as in the case of a decrease in blood volume which leads to an increase in the process of reabsorption through the renal tubules of the filter formed from the process of filtering blood in kidney [27]. The reason for the elevated serum urea levels is likely to be the adverse effects of oxidative stress that occurs in the tissues of the liver and pancreas which may have been stimulated to a large extent by diabetes mellitus[28]. Also, the reason for the high level of urea in the blood may be attributed to a decrease in the filtering ability of the kidneys and thus the accumulation of urea in the blood, and this is consistent with what was shown by each of [29,15]. The high level of creatinine in patients with DN diabetic nephropathy is one of the evidence of the disease[30,31]. The high level of urea and creatinine in the blood in patients with diabetes may indicate a kidney problem resulting from complications of diabetes[32,33]

2-Results Molecular study

The results of the ACE gene showed the presence of three genotypes in all studied samples, which are (GG, AG, and AA). When genotyping the polymorphisms of the ACE gene for the patients sample and the control group, it was found that the observed number of normal homozygous genotype AA (AA = 26) with a frequency (0.236) is the lowest and the heterozygous genotype AG (AG = 33) with a frequency (0.3) And the homozygous genotype of the mutant GG (GG = 51) and a frequency of (0.463) are the highest in infected patients. As shown in Table (4-6). The table also contains samples of the control group, where the observed number of the genotype AA (AA = 21) with a frequency of (0.5) was the highest in the control group then the genotype AG (AG = 12) with a frequency of (0.285) while the genotype GG = 9 GG) and with a frequency of (0.214) it is the lowest.The variation in these results may be due to many reasons, including what is due to genetic factors that led to the occurrence of a deletion or The variation in these results may be due to many reasons, including what is due to genetic factors that led to the occurrence of a deletion or replacement mutation in the gene causing polymorphisms of the ACE gene. Or it may be due to environmental factors including lifestyle and the nature of nutrition, which may affect the genetic material of the individual And thus increase the genetic variation and thus the predisposition to diabetic nephropathy.

Table (4-6) shows the percentages of genotypes of the ACE gene in the patients and the control group

Genotypes		**Groups**		
		AA	**AG**	**GG**
Patient (*n*=110)	**Observed(Frequency)**	26(0.236)	33(0.3)	51(0.463)
	Expected (Frequency)	23 (0.209)	43 (0.390)	44 (0.421)
Control	**Observed(Frequency)**	21(0.5)	12(0.285)	9(0.214)

(*n*=42)	**Expected (Frequency)**	22 (0.523)	14 (0.3)	6 (0.142)
***p*-value**		0.75	0.061	0.32
Chi-Square (X^2)		6.726	1.449	0.875
OR		4.21	0.53	0.66
95% CI		1.27 –13.83	0.28 – 1.49	0.33 – 1.47

As for the frequency of the alleles of the ACE gene, which were obtained from the infected group and the control group represented by the A and G alleles the proportions of the infected were as follows: G = 143 for the G allele with a frequency of (0.65) and allele A so it was A = 77 with a frequency of (0.35).

The proportions of the control group were as follows: A=56 for allele A with a frequency of (0.65) The allele G was G =28 with a frequency of (0.34) as shown in Table (4-7)

Table (4-7) shows the allelic frequency ratios of the ACE gene in the patients and the control group

rs4343			
Allele Frequency		G	A
Patient (*n*=110)	Number (Frequency)	143 (.065)	77 (0.35)
Control (*n*=42)	Number (Frequency)	28 (0.34)	56 (0.65)
Chi-Square (X^2)		7.043	
***p*-value**		0.835	
OR		1.053	2.41
95% CI		0.84-1.21	0.84- 4.21

In the current study, differences were found in the genetic distribution and allelic frequencies of ACE for G and A alleles between patients and control group. The results of the analysis of this study show that the genotypes GG and AG have a frequency of (0.463) (0.3) in the patients, respectively, which is higher than their frequency in the control group, which is (0.214) and (0.285). While the AA genotype had a frequency of (0.5) in the control group, which was higher than its frequency in the patients group (0.236). These findings are consistent with the findings of[34] . which showed a link between the GG polymorphism of ACE (rs4343) and diabetic nephropathy, indicating that the G allele conferred susceptibility to diabetic nephropathy. Also[35] indicated the association of ACE gene polymorphisms with chronic kidney disease in Han Chinese population. In a study conducted by[36] he revealed the presence of genetic polymorphisms and functional differences in the angiotensin-converting enzyme gene ACE(rs 4343) for alleles A, G, which indicates the role of both in oxidative stress and inflammation in the genetic predisposition to diabetes and diabetic nephropathy. [16] showed that the polymorphism of the ACE gene has a very close relationship with the risk of developing diabetes and ESRD associated with diabetes. In a study conducted by[37] on patients with type 2 diabetes in Egypt, it was found that there is a significant role for polymorphisms of the ACE gene in patients with type 2 diabetes in the development of the disease and the incidence of diabetic nephropathy. These are the results are consistent with the results of our study. As it was shown[38] that the genetic polymorphisms of the ACE (rs 4343) gene do not play a role in the incidence of diabetic nephropathy in patients with type 1 diabetes. The polymorphism of the ACE gene represents a genetic risk factor for diabetic nephropathy in cases of type 2 diabetes, especially in people who carry the

homozygous genotype of DD [39]. Research on polymorphisms in the ACE gene began in 1994 when Cambien and his group reported that ACE levels are under genetic control and that the association between the ACE gene and T2DN-induced diabetic nephropathy has been fully investigated but some studies yield results conflicting[40]. Genes that encode elements of the renin-angiotensin system support as determinants of susceptibility to hypertension and cardiovascular disease or both and are common in individuals with diabetic nephropathy [41]. Polymorphisms of the ACE gene are considered to be one of the causes that lead to kidney failure in patients with type 2 diabetes, especially those who carry the DD genotype[42].

CONCLUSION

The present study supports data showing the possibility of using an ACE polymorphism as a genetic risk factor for diabetic nephropathy and elevated level of ACE in the blood serum in cases with diabetes. As this study is limited with fewer cases and controls the observed genetic and allelic differences may not represent a true association. Therefore additional studies regarding gene-gene-environment interactions should be performed to estimate the overall risk of the ACE gene in causing diabetic nephropathy.

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