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Relevance of Angiotensin Converting Enzyme Gene Polymorphism 1978124 with Hypertension in Type 2 Diabetes Mellitus

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ABSTRACT

Background: *Cardio vascular disease is public in diabetes. Angiotensin-converting enzyme (ACE)2 is a recently described member of the Renin Angiotensin System, and this study examined ACE2 polymorphism is associated with hypertension and in Type 2 diabetes mellitus (T2DM).it was linked to enlarged chances hypertension was significant peripheral vascular issues, including both macro- and micro-vascular complications.* ***Aim of study:*** *To evaluate the risk of ACE2 gene polymorphism (rs1978124) in the development of Hypertension also to estimate the ACE2 levels in the recruited individuals.* ***Method:*** *the study is case control from September 2023 Variant ACE2 (rs1978124) examined in 200 Iraqi subjects diabetic. 90 hypertensive diabetic patients (case group) and 110 normotensive diabetic patients (control group) were included. Patients age>30 years old. diagnosed by physicians having T2DM with and without Hypertension. Polymerase chain reaction (PCR) used to detect the A/G alleles.* ***Results:*** *The results for rs1978124 in HT diabetic patients with, as well as in NT diabetic control were analyzed after adjusting for age, sex, and Body Mass Index was no statistically significant in all hereditary models and the minor allele frequency analysis, showed no significant differences.* ***Conclusion:*** *ACE2 gene polymorphism rs1978124 is non-linked to hypertension in T2DM Iraqi peoples.*

Keywords: ACE2; Hypertension; Renin–Angiotensin System; Type II Diabetes Mellitus.

Article Information

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INTRODUCTION

Type 2 diabetes (T2DM), the most common form of diabetes, includes a range of metabolic irregularities considered by increased blood sugar levels because of inadequate insulin secretion and/or function [(1)]. Approximately 90% of diabetes cases are attributed to type 2 diabetes mellitus (T2DM), with the remaining 10% associated with type 1 diabetes (T1DM) and gestational diabetes [(2)]. The enlarged morbidity and mortality associated with type 2 diabetes branch from its prevalent occurrence, indirect onset, and delayed detection, especially in regions with limited properties [(3)]. In Iraq, 11.5% of people of both sexes had diabetes in 2000. While it rose to 17.5% for both men and women combined in 2014, it then somewhat decreased to 17.2% at an unnamed later date. obesity is responsible for approximately 55% of type 2 diabetes cases [(4)], with the rise in young obesity from the 1960s to the 2000s highlighted as a contributing cause to the rise in type 2 diabetes in children and adolescents [(5)]. T2DM is linked to increased chances of cerebrovascular diseases, early onset coronary artery disease (CAD), and significant peripheral vascular issues, including both macro- and micro-vascular complications [(6)] These features include the effects of insulin resistance on inflammation, oxidative stress, high blood pressure, the buildup of macrophages, the development of atherosclerosis, and the functioning of blood vessels [(7, 8, 9, 10)]. Angiotensin-converting enzyme-2 (ACE-2) is a zinc-dependent metalloproteinase found on endothelial and epithelial cell surfaces. It predominantly

resides on the apical surface, where it undergoes proteolytic cleavage to form a soluble variant [11]. RNA levels of ACE2 were identified in 72 human tissues, demonstrating particularly high expression in the lungs, intestines, kidneys, testes, gallbladder, and heart [12]

The renin-angiotensin system (RAS) is a complex hormonal system that controls cardiovascular and renal homeostasis, controls extracellular fluid volume, and effects renal, The renin-angiotensin system (RAS) is a complex hormonal system that controls cardiovascular and renal homeostasis, controls extracellular fluid volume, and effects renal, immune, and neurological functions [13]. This system is made up of two pathways, which have opposing actions.

Various functions of ACE2 include: (A) Acting as a carboxypeptidase, ACE2 converts angiotensin II (Ang II) into the heptapeptide angiotensin 1-7 (Ang 1-7). (B) Serving as a receptor for SARS-CoV. (C) Interacting with amino acid transporters. Angiotensin converting enzyme 2 has been found to have significant involvement in diabetic complications affecting both small and large blood vessels [14].Research findings suggest that increased ACE activity reduces tissue perfusion in both healthy and injured animals [15].Elevated glucose levels in diabetes mellitus led to endothelial cell injury through precise pathways [16]. Causing increased activity of angiotensin converting enzyme. These complications are evident in patients with diabetes.

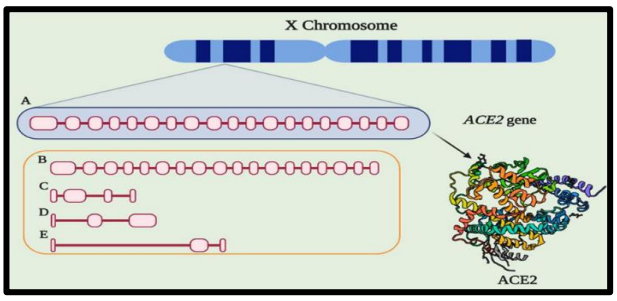

Figure 1 Show: (Structure of ACE2)

ACE2 is a hybrid protein arising from the duplication of two genes, specifically the ACE2 gene located on the X chromosome's short arm in cytogenetic band 22.2, spans 41,115 base pairs of genomic DNA. It comprises 19 exons and 18 introns [17]. The gene undergoes alternative splicing, [18] leading to five different transcripts. However, only two of these transcripts are translated into functional proteins

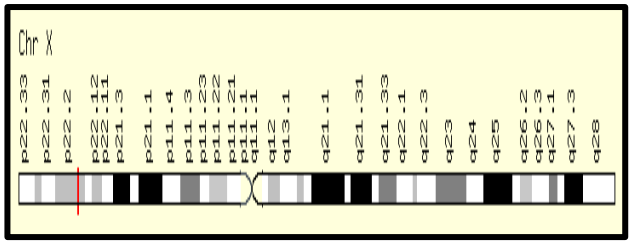

Figure 2 show: ACE2 gene location in chromosome X

The ACE2 gene is situated in a genomic region excepted from X-inactivation [20], leading to observable variations between males and females in terms of phenotype.

The first predominant variant of the ACE2 gene was denoted rs1978124. The National Center for Biotechnology Information (NCBI) provides the nucleotide sequences for single nucleotide polymorphism (SNP).

*rs1978124 This SNP (also called A1075G) is A/G within intron 1: Major allele = A, Minor allele = G. The A nucleotide is replaced by G nucleotide

CTGATGGAC[A\G] TCTCCACAC

Variations in the ACE2 gene are diverse, and alterations in enzyme function could result from these variations [21]. The ACE2 gene's position on the X chromosome is of significance concerning the gender-specific predispositions observed in coronary artery disease (CAD) and hypertension, with males displaying heightened susceptibility in contrast to females [22]. Investigations into the impact of ACE2 single nucleotide polymorphisms on cardiac vascular disease in humans yielded

varied findings. Numerous studies have investigated the connection between ACE2 single nucleotide polymorphism (SNP) and coronary artery disease and hypertension [23,24], with a focusing on SNP such as rs1978124.

METHOD

This research is a case-control, including 90 Hypertensive diabetic patients (the pathological case group) and 110 Normotensive diabetic control served as (control group). The duration of the study was from August 2023 till November 2023. Patients were taken from the Al-Najaf Center of diabetes and endocrinology situated within Al-Sadr Medical City, located in the Al-Najaf Governorate. The biochemical and genetic analyses were carried out in The Postgraduate Laboratory Department of Biochemistry, affiliated with the University of Kufa's Faculty of Medicine. Continuous variable data were summarized as mean ± standard deviation (SD). T -test was employed for comparing the control group against the group with hypertension, while ANOVA test was utilized for comparing numerical data across more than two groups. Statistical significance was determined using SPSS v. 26.0 software (SPSS Inc., Chicago, IL). The Chi-squared test ($\chi 2$) was employed to evaluate disparities in genotyping and allele frequencies between individuals HT diabetic patients and NT diabetic control subjects. Adjustment for age and body mass index (BMI) was conducted for the estimation of odds ratios (OR), confidence intervals (CI95%), and P-values. Asignificance level of $\leq$ 0.05 was applied to interpret significant findings.

Inclusion criteria includes:

1.The age of participant >30 years old.

2.Those patients are diagnosed by physicians as having type 2 diabetes with and without hypertension.

Exclusion criteria:

1. The patients less than 18 years old.
2. Patients have type 1 diabetes.

Ethical approval for the education protocol was attained from the Institutional Review Board at the Faculty of Medicine, Kufa University.

Sampling: Five milliliters of blood were drawn from each participant via peripheral vein puncture following an overnight fast. Subsequently, the blood samples were divided into two aliquots.

1-Three milliliters of blood were transferred into a plain tube and allowed to coagulate at 37°C for approximately 15 minutes, followed by centrifugation at 2000 xg for 10-15 minutes. The resulting serum was separated and stored at -20°C for the determination of phenotype parameters.

2-Two milliliters of blood were combined with EDTA in a tube for genetic analysis purposes.

The weight (Kg) and height (m) Measurements were conducted using standard methodologies, and the Body Mass Index (BMI) was derived from the formula weight (Kilograms) divided by the square of height (meters). The biochemical parameters that were measured were serum ACE2 by ELISA and Triglycerides (TG), cholesterol, and high-density lipoprotein (HDL) levels were assessed using standard enzymatic techniques. Low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) levels were calculated using mathematical formulas.

The formula for calculating the sample size in a case-control study

$$n = [(p0 \cdot q0) + (p1 \cdot q1)] \cdot (Z\alpha/2 + Z\beta)2 / (p1 - p0)2$$

$q0 = 1 - p0$ (proportion of controls not exposed)

$q1 = 1 - p1$ (proportion of cases not exposed)

$Z\alpha/2$ is the critical value from the standard normal distribution for α.

$Z\beta$ is the critical value for β.

Genotyping The extraction of DNA was achieved by the use of favorgen kits. DNA was removed from the plasma and the strengthening of the ACE2 gene was achieved using template DNA and specific primers with a master mix. The product of PCR was electrophoresed on 2% agarose (120 min and 70V) and immediately visualized under UV light. Genotypes related to ACE2 gene polymorphism rs1978124 were determined by restriction fragment length polymorphism (RFLP-PCR) technique. The amplification of the SNP was accomplished by stable primers and Taq plus master mix kit (solgent). The product of PCR was digested by a specific restriction enzyme (Biolabs.) and then analyzed by gel electrophoresis (2%).

DNA concentration and purity were determined via absorbance measurements using BIODROP. Absorbance at 260 nm corresponds to DNA concentration, with nucleic acids exhibiting maximum absorbance at this wavelength, while proteins absorb at 280 nm [25]. The A260/A280 ratio was utilized to gauge DNA purity, with a ratio falling within the range of 1.8 - 2.0 indicative of pure DNA. Additionally, absorbance at 230 nm, reflecting other contaminants, was considered by calculating the A260/A230 ratio.

Pure nucleic acid typically exhibits ratios between 1.8 - 2.2 (Assessment of Nucleic Acid Purity, 2020). Genotyping accuracy was resolute by the genotype concord between duplicate samples and was100% for the SNP.

Table 1 show: Primers of PCR amplification of ACE2 gene

SNP	Primer	Sequence
rs1978124 A\G	Forward	5-TAA CAA GTGCAA GGA TTT AGG-3
	Reverse	5-AAG CTG CAATGA ATC ATG AT-3

Table 2 Shows: Volumes of amplification reaction in traditional PCR

Reagent	Volume (25micro litter)
Reverse – primer	1.5
Forward primer	1.5
Nuclease free water	7
Taq plus Master mix	10
Genomic DNA	5

Table 3 show: The protocol of ACE2 gene polymorphisms, rs1978124 in PCR reaction

SNP	Initial denaturation	Denaturation	Annealing	Extension	Final extension	Total time
	1cycle	34 cycles& 30 Cycle			1 cycle	
Rs1978124	95°C 2 min	94°C 30 sec	52.5°C 30 sec	72°C 50 sec	72°C 5 min	88 Min

Table 4 shows: Protocols for RFLP technique of SNPs (rs1978124) of ACE2 gene

	SNP	SNP location	Region	Restriction enzyme	Temp
Rs1978124	A/G	Chromosome X	Intron1	Ava II	37 C°

Optimization of the condition of the digestion process.

* Various enzyme volumes were employed (0.5 µl, 0.2 µl, 1 µl).

* Diverse PCR product volumes were utilized (10 µl, 15 µl).

* Varying incubation durations were implemented (8 hours, 3 hours, and 4 hours).

Appropriate conditions were selected for the good digestion and are summarized in following table. The total volume was 20 µl in a 0.2 ml tube specific for PCR reaction, then centrifuged for few seconds at 2000xg, and finally incubated for the suitable time.

Table 5 shows: Appropriate volumes of the digestion reaction for rs1978124

Reagent	Volume (µl)
dd H2O	12.5
PCR product	5
10X Buffer R	2
Restriction enzyme	0.5
Time of incubation 3 hours	

RESULTS

The product of gene SNP rs1978124 A/G was found to be 471 bp. the product of the ACE2 gene of rs1978124 A/G was digested via *Ava II* restriction enzyme, 2% agarose gel was used to analyze the product. The results showed one band at 471 bp for the AA wild pattern, two bands at 305, 166 bp for the GG homozygous pattern, and three bands at 471,305 and 166 bp for the AG heterozygous pattern as presented in the following figures and,Tables.

The genetic power (36%) reflects the likelihood that this study would correctly identify an association if one truly exists. A power was relatively low, suggesting that there is a significant chance of failing to detect a true effect. the chi-square statistic indicates some level of association between Rs 1978124 and the trait under study, the P-value suggests that this result is not statistically significant at conventional levels. Furthermore, with only 36% genetic power, there is a considerable risk that true associations may go undetected in this analysis.

Genotype and Allele frequencies distribution

The result of analysis SNP (rs1978124A/G) ACE2 gene polymorphism in hypertensive (HT) diabetic patients and normotensive (NT) control subjects was analyzed using the Chi-square test across co-dominant, dominant, and recessive inheritance models. Adjusting for age, sex, and BMI, the comparison between HT patients with variant genotypes and those with the wild-type genotype (AA) revealed no statistically significant differences in the co-dominant model. Similarly, the dominant and recessive models, as well as the minor allele frequency analysis, showed no significant differences as in Table 11:

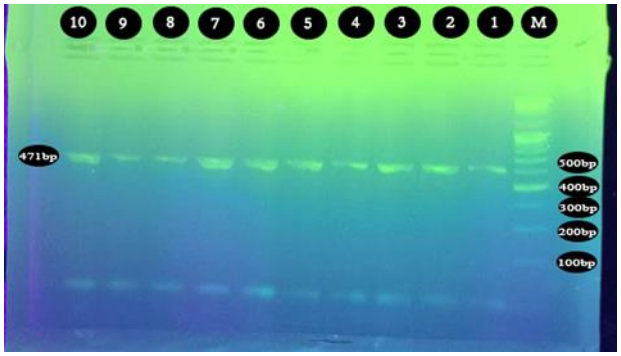

Figure 3: The PCR product of ACE2 gene analyzed for rs1978124 SNP by electrophoresis on an agarose gel.

M: Ladder of DNA 100 bp. Lanes 1-10: The PCR product with a size of amplicon 471 bp.Table 7 show: Results of ACE2 gene polymorphism, SNP rs1978124 A>G product.

Table 6 shows: the concentration and purity

DNA	Mean ± SD
DNA Conc. (µg/ml)	22.97 ± 4.03
DNA purity	1.76 ± 0.2

of DNA, Deoxyribonucleic acid; SD, Standard.

Table 7 shows: Genotype and Brand Number.

Genotype		Bands number	size (bp)
Wild type	AA	1	(471 bp)
Heterozygous	AG	3	(471, 305and 166 bp)
Homozygous	GG	2	(305 and 166 bp)

Table 8 show: Genetic power for ACE2 gene polymorphism SNP rs1978124 A/G.

SNPs	chi-square ($\chi 2$)	P-Value	Genetic power
Rs 1978124	5.4	0.07	36%

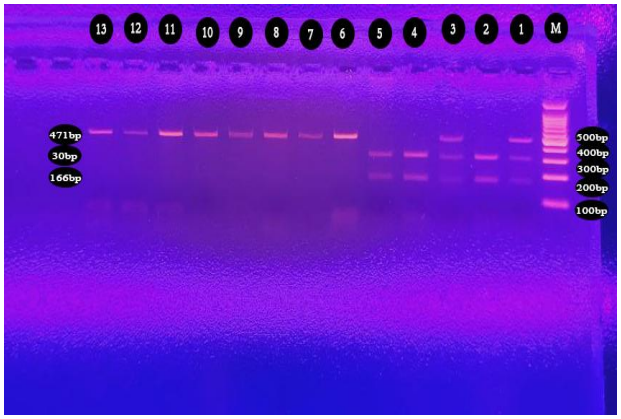

Figure 4: The RFLP product of rs1978124 SNP of ACE2 gene polymorphism after digestion byAva II enzyme. *M: DNA ladder 100 bp. Lanes 6-13:(AA) wild homozygous genotype 471. Lanes Iand 3: (AG) heterozygous genotype 471 ,305 and 166bp.Lanes 2,4 and 5:(GG) genotype 305 and 166bp.*

Table 9 shows: Hardy-Weinberg equilibrium in the " SNP (rs1978124A/G) polymorphism genotype: principle is expressed mathematically by the equation "p^2 + 2pq + q^2 = 1, [26] where "p" and "q" denote the frequencies of alleles. The calculation of HWE was performed utilizing the web-Assotest. ACE2 gene polymorphism (rs1978124 A>G) was deviated from HWE.

Gene\SNP Genotype	Gene\SNP Genotype	Controls			
		Observed Genotype	Expected Genotype	P-Value	Chi Square (X2)
ACE2 gene rs1978124	AA	102	90.25	0.00001	15.17
	AG	6	9.5		
	GG	2	0.25		

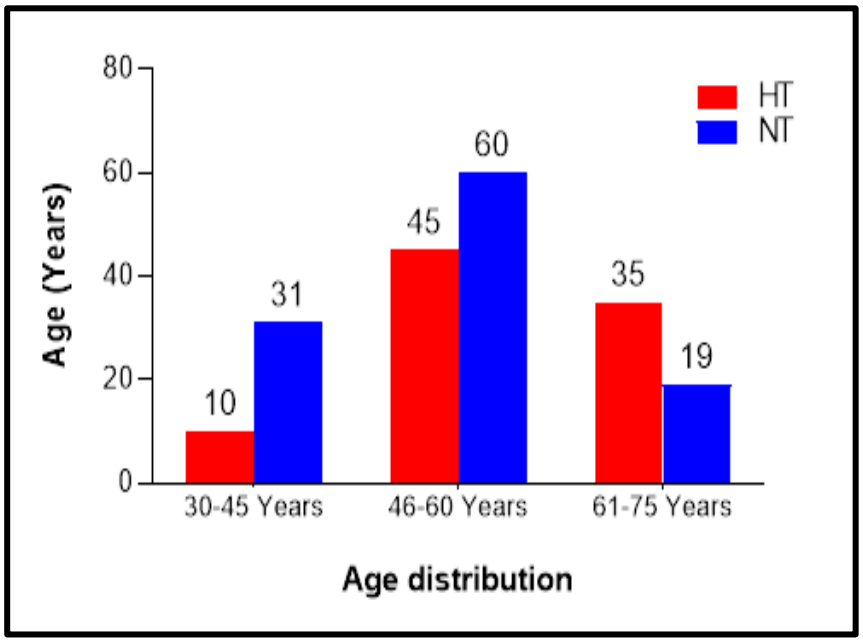

Figure 5 Shows: The distribution of age in studied groups.

Table 10 Shows Socio -demographic Characteristics of Study Groups.

	HT (n= 90)	NT (n=110)	P-Value
Age (years), (mean ±SD)	57.40 ± 8.80	51.5 ± 9.83	0.001*
BMI (mean ±SD)	30.46 ± 5.21	29.47 ± 4.37	0.153
FBS (mg/dL), (mean ±SD)	188.27 ± 78.86	192.16 ± 80.65	0.731
HbA1c (%), (mean ±SD)	8.29 ± 2.06	8.65 ± 3.09	0.669
TC (mg/dL), (mean ±SD)	151.43 ± 40.87	158.22 ± 45.70	0.270
TG (mg/dL), (mean ±SD)	236.86 ± 106.94	227.60 ± 121.45	0.568
HDL (mg/dL), (mean ±SD)	34.67 ± 5.96	33.91 ± 5.77	0.365
VLDL (mg/dL), (mean ±SD)	48.11± 20.56	47.10 ± 23.73	0.747
LDL (mg/dL), (mean ±SD)	70.28 ± 41.93	79.79 ± 44.40	0.124
ACE2 (ng/mL), (mean ±SD)	8.52 ± 4.74	7.11 ± 3.83	0.341

*HT, hypertensive; NT, normotensive; n, Number of participants; BMI, Body Mass Index; FBS, fasting blood glucose; HbA1c, Haemoglobin A1; TC, total cholesterol; TG, Triglyceride; HDL, High Density lipoprotein; VLDL, very low-Density lipoprotein; LDL-C, Low Density lipoprotein; ACE2; Angiotensin – converting enzyme 2; SD, Standard Deviation; *, P< 0.05.*

Table 11 show: Distribution of genotypes and allele frequencies of ACE2 gene polymorphism (rs1978124 A>G) in HT and NT diabetic groups.

Models	HT (n= 90)	NT (n=110)	Basic OR (CI 95%)	P- value	Adjusted OR (CI 95%)	P- value
Codominant						
AA	87	103	Ref.	-	-	-
AG	1	6	2.30 (0.20 -26.50)	0.480	1.90 (0.16 – 23.20)	0.110
GG	2	1	0.19 (0.02 – 1.60)	0.130	0.20 (0.02 – 1.90)	0.170
Dominant						
AA	87	103	Ref.	-	-	-
GA+GG	3	7	0.50 (0.12 – 2.01)	0.320	0.50 (0.13 – 2.10)	0.310
Recessive						
AA+GA	88	109	Ref.	-	-	-
GG	2	1	2.40 (0.20 – 27.4)	0.460	2.20 (0.10 – 26.70)	0.510
Frequency of Alleles						
A	0.97	0.96	Ref.	-	-	-
G (MAF)	0.03	0.04	1.15 (0.35 – 3.68)	0.814	1.42 (0.37 – 5.34)	0.604

*HT, hypertensive; NT, normotensive; n, Number of participants; OR, odds ratio; MAF, minor allele frequency; Ref., Reference; *, P>0.05.*

DISCUSSION

In order to minimize the effects of T2DM on the health care system and its effects on community vitality, scientists have focused a great deal of research effort on the causes and consequences of the disease in humans among different populations worldwide. Studies continue to identify individuals who are very likely to develop consequences of T2DM, such as hypertension. Essential high blood pressure is known as the main reason for the universal death of cardiovascular illness [27]. The current research explored potential associations between polymorphisms in the ACE2 gene and various physiological and pathological conditions. Rs1978124 SNP with hypertension in the T2DM Iraqi population**.**

The genetic power of the ACE2 gene polymorphisms (rs1978124 A>G) was demonstrated to be 36%, suggesting supporting evidence of the results as a level of 80% is believed to be enough in sufficient to demonstrate a reasonable decision (osse.bii.a-star.edu.sg). However, these findings may provide useful information for planning future research projects analyzing ACE2 gene polymorphisms in hypertensive diabetic patients**.** One ACE2 gene polymorphism (rs1978124 A>G) was identified to deviate from the Hardy-Weinberg equilibrium. Numerous factors, including indiscriminate mating, mutations, and the size of the recruited sample, could contribute to the discrepancy [27]. The most likely cause is the relatively small sample size, of 200 participants recruited in the current case-control study**.** The data regarding the rs1978124 A>G polymorphism did not show a significant connotation with the illness**.**

To elucidate the molecular role of the examined single nucleotide polymorphism (SNP) in the pathogenesis of hypertension, it is crucial to discuss the influence of ACE2 on hypertension and the specific effects of this SNP on the ACE2 gene**.**

ACE2 is a mono carboxy peptidase that exhibits high activity in breaking down of apelin-13 and dynorphin A 1-13. The breakdown of angiotensin II (Ang II) to angiotensin 1-7 (Ang 1-7) is the most chief biological action of ACE2, and the catalytic efficiency of Ang II is 400 times higher than that of Angiotensin I (Ang I) [28] Therefore, ACE2 may inhibit Ang II vasoconstrictor action through its degradation and offset Ang II activities by forming Ang 1-7, which has antifibrotic and vasodilatory activity at Ang 1-7 Mas receptor [29]

Most evidence suggests that ACE2 may serve as an undesirable manager of the activated renin-angiotensin-aldosterone system (RAAS) and that ACE2 function loss is associated with hypertension, most likely due to diminished generation of the powerful vasodilator Ang 1-7 [29**.(**

The rs1978124 SNP is located on intron1, the single nucleotide polymorphism rs1978124 involves a substitution where adenine (A) is replaced by guanine (G). Regarding ACE2 SNP, these genetic variations may influence the quantity and function of the ACE2 protein. Specifically, amino acid changes associated with these polymorphisms, particularly rs1978124, could alter the structure and activity of the resultant ACE2 protein [30], It may be involved in the ACE2 mRNA stability (conformation), mRNA splicing efficiency (intron splicing enhancer or silencer element), and control of post transcriptional modification, recent studies have at least partially verified that ACE2 expression alterations were observed at the protein level rather than the mRNA level [31]

Previous research provides support for this interpretation. Pinheiro et al. (2019) [29] and Pan et al. (2018) [31] did not find a significant relationship for the genotypes of ACE2 polymorphisms with serum lipid profile

concentrations in Brazilian and Chinese hypertensive diabetic patients, respectively.

It is essential to acknowledge the limitations of this study. Firstly, the sample size of hypertension patients and control individuals is relatively small. Secondly, factors related to socioeconomic behavior, such as lifestyle, smoking, and dietary habits, may introduce confounding variables. Consequently, further research is required to address and control for these potential confounders.

CONCLUSIONS

1. 1.ACE2 gene polymorphism, rs1978124, is not linked to the hypertension in T2DM Iraqi population.
2. 2.Differences in serum lipid concentrations
3. are not influenced by the ACE2 gene polymorphism rs1978124 genotype.

RECOMMENDATIONS

1. 1.Larger sample size is required for studying relevance of ACE2 gene SNP (rs1978124) to hypertension
2. 2.More SNPs are demanded to be studied in ACE2 gene in Iraq to illustrates their prevalence or association with hypertension in T2DM Iraqi population.
3. 3.Implication in the gene-gene interaction with various genes that linked to the occurrence of hypertension in T2DM Iraqi population.
4. 4.In the latest forensic statistics in Iraq, it was proven that heart disease is the first and main cause of the increase in the number of deaths. Therefore, it is necessary to focus on confronting this cause by establishing advanced research and medical centers (one of scientific reports in Iraq).

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