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Relevance of Angiotensin Converting Enzyme Gene Polymorphism rs2285666 with Hypertension in Type 2 Diabetes Mellitus

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ABSTRACT

Background: *Cardiovascular disease is common in diabetes, and is connected with activation of the renin-angiotensin system (RAS). Angiotensin-converting enzyme (ACE)2 is a recently described member of the RAS, and this study investigated ACE2 polymorphisms associated with hypertension and T2DM. It was linked to increased chances hypertension was a significant peripheral vascular issue, including both macro- and microvascular complications.* ***Aim of the study:*** *To evaluate the risk of ACE2 gene polymorphisms (rs2285666) in the development of hypertension and also to estimate the ACE2 levels in the recruited individuals and relevance with the analyzed SNP* ***Method:*** *Variant ACE2 (rs2285666) examined in 200 Iraqi subject's diabetics with and without hypertension. 90 HT diabetic patients (case group) and 110 NT diabetic patients (control group) were included. Patients aged>30 years old and patients diagnosed by physicians as having T2DM with and without hypertension.* ***Exclusion criteria include*** *patients who have T1DM or need insulin injections. assessments were determined for each patient: age, sex, body mass index (BMI), diabetes, blood pressure, (FBS), (HbA1c), (Chol), (LDL), (HDL), (TG) andACE2serum conc. Polymerase chain reaction (PCR) is used to detect the G/A alleles. (Chi-squared and t-test) (odds ratios) were applied to determine the association between G/A polymorphism and hypertension. P<0.05 was considered statistically significant.* ***Results:*** *Results of analysis of the rs2285666 G>A pointed out a significant association with the development of hypertension. After adjusting for age, sex, and BMI in the co-dominant model, heterozygous (GA) significantly distended the danger of hypertension by three folds with deference to those of the wild homozygous (GG) (OR = 3.11, CI 95% = 1.04-9.34, P = 0.042). In the same way, the homozygous (AA) genotype suggestively raised the risk of hypertension by onefold (OR= 1.49, CI 95%; 0.82-3.21, P= 0.039). Conversely, both dominant and recessive inheritance patterns were associated with an increased risk in diabetic patients with HT. The dominant model showed an odds ratio (OR) of 1.56 (95% confidence interval CI: 1.72-4.21, P = 0.001), while the recessive model showed an OR of 2.13 (95% CI: 1.74-6.21, P = 0.021). The minor allele (A) frequency in HT patients (0.17) was found to be significantly (P=0.022) decreased when linked to the NT diabetic control groups.* ***Conclusion****: ACE2 gene polymorphism, mutant homozygous (AA) and heterozygous (GA) genotypes, rs2285666, are associated with hypertension in T2DM Iraqi population with a 2 folds risk factor to develop the disease as well as differences in serum lipid concentrations are not influenced by the ACE2 gene polymorphism rs2285666 genotypes.*

Keywords: Angiotensin-Converting Enzyme 2 Gene; Blood Pressure; Hypertension; Renin-Angiotensin System; Type 2 Diabetes Mellitus.

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INTRODUCTION

Type 2 diabetes (T2DM), the most common form of diabetes, includes a range of metabolic irregularities characterized by increased blood sugar levels because inadequate of insulin secretion and/or function [1]. Approximately 90% of diabetes cases are attributed to T2DM, with the remaining 10% associated with type 1 diabetes (T1DM) and gestational diabetes [2]. The increased 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]. A significant association is evident between Type 2 Diabetes Mellitus (T2DM) and cardiovascular disease (CVD). Due to their effects on multiple bodily systems. (T2DM) is linked to increased chances of cerebrovascular diseases, early onset coronary artery disease (CAD), and significant peripheral vascular issues, including both macro and microvascular complications [6]. These factors 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]. The subsequent sections thoroughly outline the primary risk factors for cardiovascular complications in type 2 diabetes, along with their interconnections.

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 regulates 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.ACE2 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, demonstrated in Fig.1. 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, which 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 [19]. Fig.2.

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 rs2285666. The National Center for Biotechnology Information (NCBI) provides the nucleotide sequences for these two single nucleotide polymorphisms (SNP) as outlined (NCBI, 2020).

* rs2285666

This SNP (also called G8790A) is G/A within intron 3:

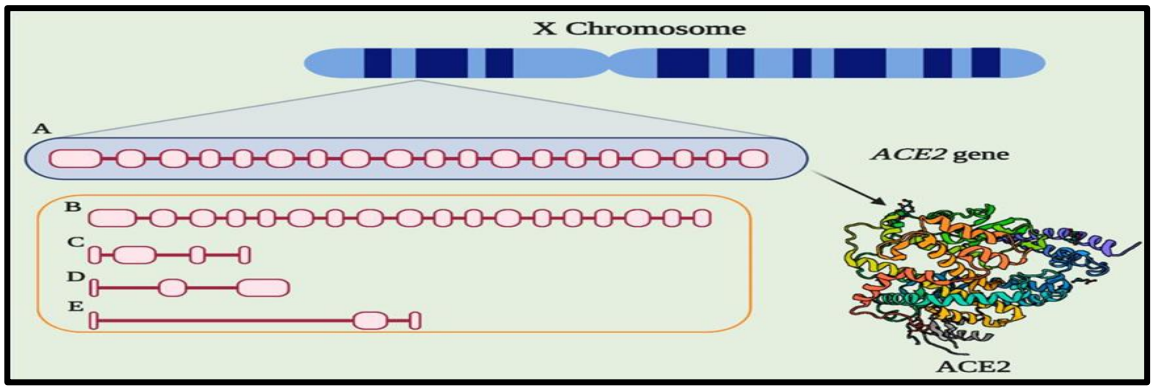
Major allele =G, Minor allele =A

The G nucleotide is replaced by A nucleotide at nucleotide + 4 of intron 3 (www.ncbi.nlm.nih.gov).

GAACCAGGT[G\A] CTACTAATT

This polymorphism has been linked to conditions such as hypertension, renal disease, and diabetes mellitus [21,22]. The single nucleotide polymorphism (SNP) located at G8790A in intron 3 was determined to lack correlation with essential hypertension across both male and female participants [23]. Some studies have associated genetic variations in the ACE2 gene with cardiovascular disease and diabetes mellitus.

Figure 1: Structure of ACE2



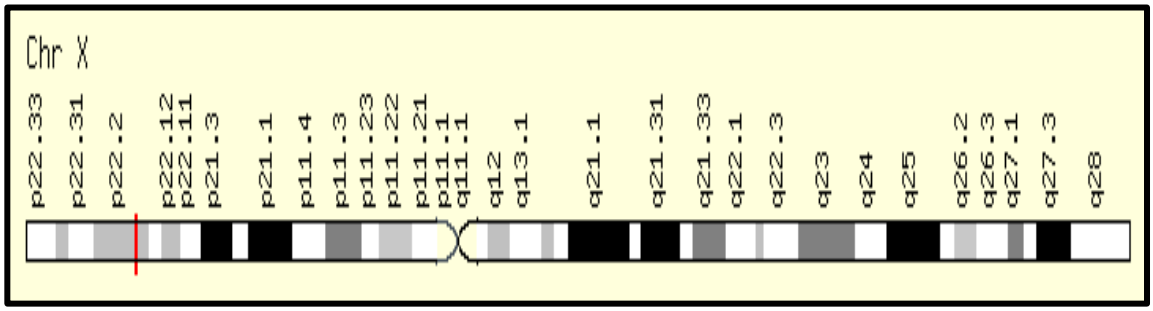


Figure 2: ACE2 gene location in chromosome X.

PATIENTS AND METHODS

This research is a case-control, including 90 hypertensive diabetic patients (the pathological case group) and 110 normotensive diabetic patients served as (a control group). The duration of the study was from August 2023 till November 2023. Patients were taken from the Al-Najaf Center of Cardiac Surgery and Transcatheter therapy as well as the specialized facility for diabetes and endocrinology situated within Al Sadder 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. ***Ethical approval*** for the study protocol was obtained from the Institutional Review Board at the Faculty of Medicine, Kufa University.

Collecting of Samples

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.

BMI measurement

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).

Biochemical parameters measurement

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 extraction of DNA

The extraction of DNA was achieved by the use of favorgen kits. Genotype related to ACE2 gene polymorphisms, rs1978124 was determined by RFLP-PCR technique. The amplification of this 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 [(24)]. 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; Lucina-Aguilar G & Carrillo Avila JA, 2016; Assessment of Nucleic Acid Purity, www.nanodrop.com). Genotyping accuracy was determined by the genotype concordance between duplicate samples and was100% for the SNP.

Table 1: Primer of PCR amplification of ACE2 gene for rs2285666.

SNP	Primer	Sequence
rs2285666 G\A	Forward	5-CATGTGGTCAAA AGGATATCT-3
	Reverse	5-AAAGTA AGGTTGGCAGACAT-3

Table 2: 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: The protocol of ACE2 gene polymorphisms, rs2285666, in PCR reaction

SNP	Initial denaturation	Denaturation	Annealing	Extension	Final extension	Total time
	(1cycle)	(34 cycles) & (30 Cycle)			(1 cycle)	
Rs2285666	95°C 2 minutes	94°C 30 second	53.9°C 30 second	72°C 45 second	72°C 7 minutes	92 Minute

Table 4: Protocols for RFLP technique of SNPs (rs2285666) of ACE2 gene

	SNP	SNP location	Region	Restriction enzyme	Temperature
Rs2285666	G\A	Chromosome X	Intron3	A1u I	37 Cº

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: Appropriate volumes of the digestion reaction for rs2285666

Regent	Volume (µl)
ddH2O	12.5
PCRproduct	5
10X Buffer Tango	2
Restriction enzyme	0.5
Time of incubation 8 hours	

RESULTS

DNA was extracted from the blood and the amplification of the ACE2 gene was performed using template DNA and specific primers with a master mix. The product of PCR was electrophoresed on 2% agarose (120 min and 75V) and immediately visualized under UV light. The amplification product of gene SNP rs2285666 G/A was found to be 466 bp. the product of the ACE2 gene of rs2285666 G/A was digested via *Alu I* restriction enzyme and 2% agarose gel was used to analyze the product. The results showed one band at 466 bp for the GG wild pattern, two bands at 281, 185 bp for the AA homozygous pattern, and three bands at 466,281 and 185 bp for the GA heterozygous pattern presented in the following figures and tables: The concentration and purity of the extracted DNA were calculated by measuring the ratio of absorption at two wavelengths, A260/A280.

Table 6: concentration and purity of DNA.

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

DNA, Deoxyribonucleic acid; SD, Standard Deviation.

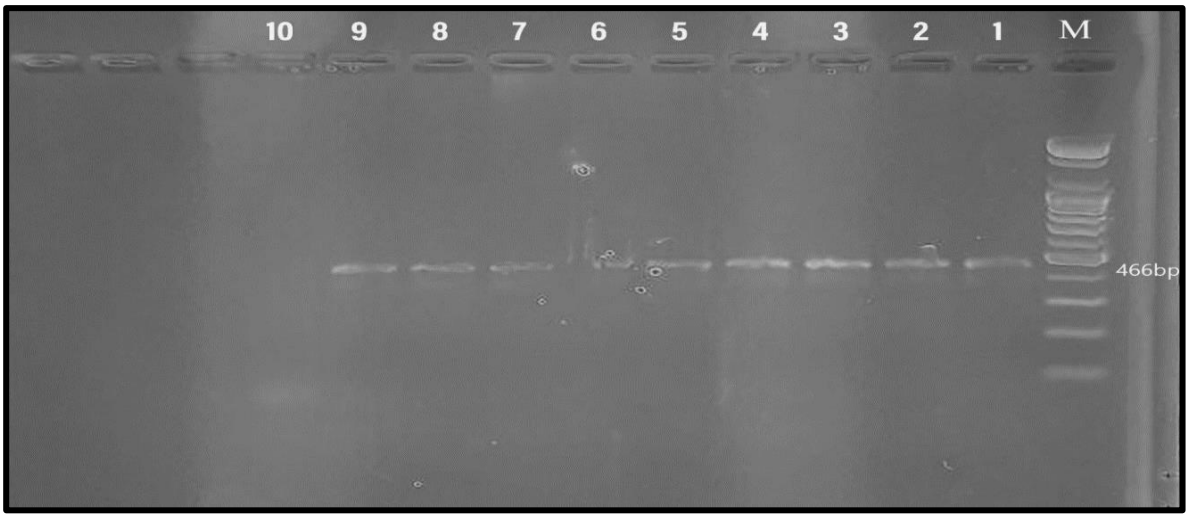


Figure 3: The PCR product of ACE2 gene analyzed for rs2258666 SNP by electrophoresis on an agarose gel. M: Ladder of DNA 100 bp Lanes 1-9: The PCR product with a size of amplicon 466 bp.

Table 7: Results of ACE2 gene polymorphism SNP rs2285666 G>A product

Genotype	Bands number	size (bp)
GG	1	(466 bp)
GA	3	(466, 281 and 185 bp)
AA	2	(281and 185 bp)

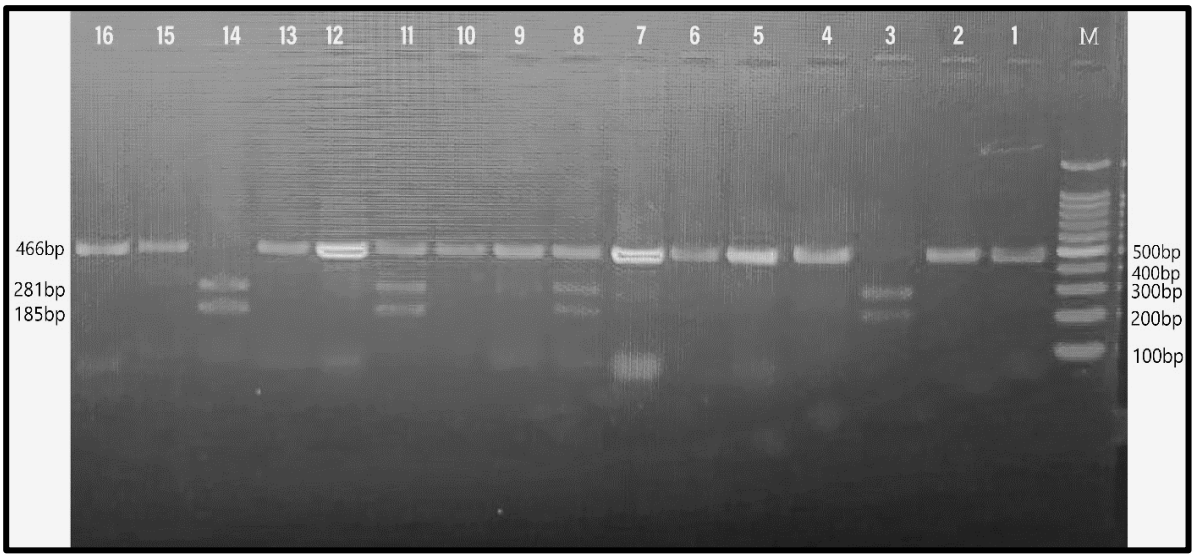


Figure 4: The RFLP product of rs2285666 SNP of ACE2 gene polymorphism after digestion by *Alu I* enzyme. M: DNA ladder 100 bp. Lanes 8 and 11: (GA) genotype 466 ,281 and 185bp. Lanes 3 and 14: (AA) genotype 281 and 185bp. Lanes 1,2,4,5,6,7,9,10,12,13,15 and 16: (GG) genotype 466.

Table 8: Hardy-Weinberg equilibrium in the " SNP (rs2285666G/A) polymorphisms genotypes.

Gene\SNP Genotype	Gene\SNP Genotype	Controls			
		Observed Genotype	Expected Genotype	P-Value	Chi Square (X2)
ACE2 gene rs2258666	GG	93	84.64	0.38	0.77
	GA	17	14.72		
	AA	0	0.64		

Genotypes and Allele frequencies of rs2285666 G>A

Results of analysis of the rs2285666 G>A pointed out a significant association with the development of hypertension. After adjusting for age, sex, and BMI in the co-dominant model, heterozygous (GA) significantly distended the danger of hypertension by three folds with deference to those of the wild homozygous (GG) (OR = 3.11, CI 95% = 1.04-9.34, P = 0.042). In the same way, the homozygous (AA) genotype suggestively raised the risk of hypertension by onefold (OR= 1.49, CI 95%; 0.82-3.21, P= 0.039). Conversely, both dominant and recessive inheritance patterns were associated with an increased risk in patients with HT. The dominant model showed an odds ratio (OR) of 1.56 (95% confidence interval CI: 1.72-4.21, P = 0.001), while the recessive model showed an OR of 2.13 (95% CI: 1.74-6.21, P = 0.021). The minor allele (A) frequency in HT patients (0.17) was found to be significantly (P=0.022) decreased when linked to the NT diabetic control groups**, Table 9.**

Table 9: 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						
GG	67	80	Ref.	-	-	-
GA	14	7	2.38 (0.91 -6.25)	0.077	3.11 (1.04 - 9.34)	0.042*
AA	9	23	2.14 (0.92 – 4.93)	0.074	1.49 (0.82 – 3.21)	0.039*
Dominant						

GG	**67**	**80**	**Ref.**	**-**	**-**	**-**
GA+AA	**23**	**23**	**1.23 (1.42 - 4.03)**	**0.001***	**1.56 (1.72 - 4.21)**	**0.001***
Recessive						
GG+GA	**81**	**87**	**Ref.**	**-**	**-**	**-**
AA	**9**	**23**	**2.55 (0.97 – 5.11)**	**0.054**	**2.13 (1.74 – 6.21)**	**0.021***
Frequency of Alleles						
G	**0.83**	**0.75**	**Ref.**	**-**	**-**	**-**
A (MAF)	**0.17**	**0.25**	**1.46 (0.89 – 2.39)**	**0.126**	**1.75 (1.09 – 3.42)**	**0.022***

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

Table 10: Biochemical characteristics of HT patients with genotypes of (rs2285666 G>A) SNP in ACE2 gene under a co-dominant model.

Parameters	GG (n=67)	GA (n= 14)	AA (n=9)	P-Value
Age	**53.67 ± 10.07**	**57.33 ± 6.59**	**56.90 ± 8.46**	**0.220**
BMI	**29.60 ± 4.61**	**32.50 ± 4.55**	**30.76 ± 5.92**	**0.150**
FBS (mg/dL)	**190.48 ± 78.77**	**200.33 ± 90.35**	**185.62 ± 85.99**	**0.890**
HbA1c (%)	**8.60 ± 7.05**	**7.65 ± 1.33**	**8.49 ± 6.52**	**0.810**
TC (mg/dL)	**157.71 ± 43.98**	**146.33 ± 39.79**	**138.38 ± 39.60**	**0.310**
TG (mg/dL)	**235.06 ± 117.36**	**163.33 ± 71.31**	**234.38 ± 104.22**	**0.180**
HDL (mg/dL)	**33.97 ± 5.69**	**39.93 ± 5.30**	**34.14 ± 6.37**	**0.010***
VLDL (mg/dL)	**48.03 ± 22.68**	**34.75 ± 14.83**	**48.57 ± 21.06**	**0.250**
LDL (mg/dL)	**78.30 ± 43.43**	**57.38 ± 32.01**	**60.52 ± 44.49**	**0.100**
ACE2 (ng/mL)	**6.93 ± 4.13**	**7.03 ± 4.86**	**6.10 ± 3.35**	**0.690**

***BMI**, Body Mass Index; **FBS**, fasting blood glucose; **HbA1c**, Hemoglobin 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.*

DISCUSSION

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 [25]. "The current research explored potential associations between polymorphisms in the ACE2 gene and various physiological and pathological conditions." (rs2285666 G>A) with hypertension in the T2DM Iraqi population. The genetic power of ACE2 gene polymorphism (rs2285666 G>A) was demonstrated to be 90 % suggesting a supporting evidences of the results as a level of 80% is believed to be 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 polymorphism in hypertensive diabetic patients.

Examined SNP (rs2285666 G>A) of the control group was demonstrated to be compatible with Hardy-Weinberg equilibrium, providing evidence that the genotype allocation in the Iraqi population remains constant from the forefather to following generation. Therefore, any changes to this distribution in hypertensive individuals will explain their role in the pathogenesis of this disease [26]. Data of rs2285666 G>A SNP in ACE2 gene demonstrated a significant association of this SNP with the occurrence of hypertension in T2DM patients.

In individuals carrying the mutant homozygous genotype (AA) and heterozygous genotype (GA), there is a twofold increased risk of developing hypertension compared to those with the reference genotype (GG).

Adjustment of age, sex, and BMI have little effect on the results by increased risk factor of 3 folds. In the Iraqi population with type 2 diabetes mellitus (T2DM), both dominant and recessive genetic styles are associated with a twofold increased risk of developing hypertension. Specifically, carriers of the minor allele frequency (MAF) variant A have a doubled risk, suggesting the involvement of the rs2285666 polymorphism in the development of hypertension in this group. To elucidate the molecular role of the examined single nucleotide polymorphisms (SNP) in the pathogenesis of hypertension, it is crucial to discuss both the influence of ACE2 on hypertension and the specific effects of that 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 chief biological action of ACE2, and the catalytic efficiency of Ang II is 400 times higher than that of Angiotensin I (Ang I) [27]. 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 [28]. Most evidence suggests that ACE2 may serve 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 [28].

The rs2285666 SNP is located on intron 3 involves a substitution where guanine (G) is replaced by adenine (A) these genetic variations may influence the quantity and function of the ACE2 protein. Specifically, amino acid changes associated with this polymorphism, particularly rs2285666 could alter the structure and activity of the resultant ACE2 protein [29] 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 [27]. In the ACE2 polymorphism rs2285666 (G>A), the substitution of guanine (G) with adenine (A) influences alternative splicing and alters ACE2 gene expression. This variation leads to a reduction in the serum levels of angiotensin 1–7 (Ang 1–7) [30]. The observed association between the A allele of the rs2285666 G>A single nucleotide polymorphism (SNP) and hypertension in an Iraqi population sample aligns with prior research findings. Pinheiro et al. (2019) [28] have specified that the (A) allele of ACE2rs2285666 G>A is associated with hypertension in Brazilian patients. Pan et al. (2018) [26] reported a correlation between the GA genotype and the prevalence of hypertension in the Chinese population. on the hands, Benjafield et al. (2004) [31] investigated the association between the AA genotype and hypertension risk, finding that Australian individuals with this genotype did not exhibit an increased risk of developing hypertension.

CONCLUSIONS

1. ACE2 gene polymorphism, mutant homozygous (AA) and heterozygous (GA) genotypes, rs2285666, are associated with hypertension in T2DM Iraqi population with a 2 folds risk factor to develop the disease.

2. Differences in serum lipid concentrations are not influenced by the ACE2 gene polymorphism rs2285666 genotypes.

RECOMMENDATIONS

1. Larger sample size is required for studying relevance of ACE2 gene SNPs (rs2285666 and rs1978124) to hypertension.

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. Implication in the gene-gene interaction with various genes that linked to the occurrence of hypertension in T2DM Iraqi population.

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 center

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