Original Research

Relationship of angiotensin converting enzyme (I/D) polymorphism (rs4646994) and ischemic heart disease in Iraqi patients with type 2 diabetes mellitus

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Abstract

Insertion deletion (I/D) polymorphism (rs4646994) in the angiotensin-converting enzyme (ACE) has a substantial effect on Coronary Heart Disease (CHD). The objective of this study was to investigate the association between the *ACE* gene polymorphism and CHD in Iraqi patients with and without Type-2 Diabetes Mellitus (T2DM). The amplification of an Alu repetitive element in an intron of the *ACE* has shown three potential genotypes of I/I and D/D as homozygous, and I/D as heterozygous. A total of 217 individuals participated in this study, and they were divided into three groups; Group 1 included 86 patients who had CHD with T2DM, Group 2 included 78 patients who had CHD without T2DM, and Group 3 included 53 age and sex-matched healthy individuals (as a control group). Genotyping of *ACE* (I/D) gene was performed using polymerase chain reaction (PCR) technique. Our data showed a significantly high D/D genotype frequency in the *ACE* in CHD patients compared to the healthy individuals (OR=4.39, CI 1.57223–12.2749, P<0.0048), whereas I/D genotype was not affected (OR=1.1009, CI 0.5540–2.1878). This suggested that the D/D genotype is an independent risk factor for CHD in Iraqi patients with and without T2DM. We concluded that the D/D genotype is implicated as a risk factor for CHD patients, in the Iraqi population. However, a larger sample size is needed to monitor the CHD patients and validate this study.

Keywords: Angiotensin converting enzyme, Coronary heart disease, Ischemic heart disease 2 Diabetes Mellitus.

Introduction

In coronary heart disease (CHD), fat accumulates at the end of the atrium due to an unusual metabolism of this substance. These precipitated fats cause reduction of the arteries' cavity, retarded blood flow, and consequently leads to ischemic heart failure along with clinical symptoms such as angina. Because of the high morbidity and death rate, CHD has become one of the most dangerous cardiovascular diseases threatening people's lives [1, 2]. Approximately, one third or more of all deaths in people of 35 years old and older are due to this disease [3]. The most common cause of ischemia is atherosclerosis, which is the consequence of dyslipidemia, causing a local decrease in blood-flow through the heart muscle, as well as insufficient heart muscle perfusion provided by the coronary artery [4]. It occurs to individuals of all ages, but is more common in the elderly, with males being more susceptible than females. However, other common risk factors are smoking, family history, high blood pressure, excessive weight gain, diabetes, high alcohol consumption, little or no exercise, stress, and high blood fat [5]. Symptom

of Ischemic Heart Disease (IHD) includes angina (severe chest pain during exertion) and heart palpitation due to decrease or lack of exercise. Diagnosis of the IHD is done through an electrocardiogram, cardiac stress test, blood tests, or coronary angiography. For symptomatic patients, a stress echocardiogram is used to diagnose obstructive coronary artery disease [6]. The patient's age, gender, coronary risk factors, and the nature of chest pain are also important factors that determine the risk of CHD and facilitate the diagnosis. Hence, the ECG exercise test is a diagnostic test that needs to be performed accurately [7]. Partial or complete blocked arteries cause angina or heart attack, respectively, due to the gradual death of heart cells [8]. The actual numbers of these anomalies vary from one country to other [9]. Each year, approximately 790,000 adults suffer from myocardial infarction (MI), and 210,000 of them suffer from frequent heart attacks [10]. Somatic angiotensin-I converting enzyme (sACE) plays an essential role in regulating the blood pressure and electrolyte fluid balance. It is a zinc protease that cleaves angiotensin-I (AngI), bradykinin, and a large group of other signal peptides [11]. The ACE is expressed in cells in the bone marrow and encoded angiotensin-converting enzyme (ACE). It converts angiotensin I to angiotensin II active peptide, leading to increased hematopoietic stem cells [12]. Single nucleotide polymorphisms (SNPs) of renin-angiotensin system (RAS) genes such as ACE, has been shown to be linked strongly to cardiovascular disease (CVD) [13]. In CVD, one of the most extensively studied genes was the ACE and I/D polymorphism of ACE gene that strongly associated with the ACE activity [14].

Materials & Methods

Subjects

This study was designed as a case-control survey comprising of 217 male subjects classified into three groups. The group I had 86 patients who had IHD with T2DM, the group II had 78 patients having IHD without T2DM, and the group III had 53 apparently-healthy individuals without any diseases, which were used as a control group. Samples were collected from Al-Hussein Medical City, Kerbala from December 1/2018 to July 31/2019. The biochemical parameter determinations and genetic analysis were performed in the Department of Biochemistry, College of Medicine, University of Kerbala and Laboratory of Al-Hussein Medical City. The exclusion criteria were: (1) Female; (2) Type 1 diabetes mellitus; (3) Patients treated with catheterization; (4) Children. The inclusion criteria were as follows: (1) The selected patients diagnosed with one of the following diseases: myocardial infarction (MI) with T2DM, MI without T2DM, unstable angina with T2DM, unstable angina without T2DM; (2) Adult male patients; (3) Before performing catheterization; (4) With or without risk factor: smoking, family history, hypertension and T2DM. Informed consent was taken from all subjects according to the Ethical Committee of the Kerbala Medical College, who approved the study. Blood was drawn (5 ml) through vein puncture from all participants. The collected blood was divided into three parts: (1) One milliliter of blood used for molecular analysis (DNA extraction), was collected in the EDTA containing tube, (2) One milliliter used for HbA1c analysis in the EDTA containing tube, (3) Three milliliters of blood collected in a gel tube for biochemical analysis.

The *ACE* (I/D) polymorphism (rs4646994) detection by polymerase chain reaction

DNA was extracted from the blood samples by the genomic DNA extraction Kit (Geneaid Biotech Ltd., UK), according to the manufacturers' protocol. Concentration and purity of isolated DNA was measured by a BioDrop (UK). The existence of the I and D alleles of the ACE was detected by PCR, according to the essay described by Zmorzynski *et al.* [12]] The sequence of forward primer was 5'-CTG GAG ACC ACT CCC ATC CTT TCT-3', and the reverse primer was 5'-GAT GTG GCC ATC ACA TTC GTC AGAT-3'. PCR reaction was performed with 100 ng DNA in a final volume of 25 μl, containing 12.5 μl mastermix (Promega, Madison, WI, USA), 1 μl of each primer

(10 pmol). The PCR program for determining the genotype of the ACE (I/D) (rs4646994) was done in a thermal cycler (Biometra, Germany), with 95˚C initial denaturation for 5 minutes, and 35 cycles of denaturation at 95˚C for 30 seconds, annealing at 60˚C for 30 seconds, and extension at 72˚C for 40 seconds, and finally, one cycle at 72˚C for 5 minutes. Lastly, 7 μl of amplicon was separated by electrophoresis on a 2% agarose gel, containing safe stain and finally visualized by a transilluminator. The I allele manifested as a 490 bp band, while the D allele was seen as a 190 bp band of DNA, but I/D genotype, illustrated as two bands, appeared at 490 bp and 190 bp as demonstrated in Figure 1.

D/D genotype showed a single band with 190 bp, lane 2,4,7 homozygous; I/I genotype had a single band with 490 bp, lane 1,3,5,9 homozygous; and I/D genotype demonstrated two bands 190 and 490 bp, lane 6, 8 heterozygous; L is the indicated ladder.

Statistical analysis

A student t-test was applied to biochemical variables, in comparison with IHD patients with polymorphism, and Chi-square test for categorical variables, using SPSS v.25. Fischer's exact test was used to assess the relationship of the ACE I/D polymorphism with the risk factor. Also, the quantitative information was shown as the frequency or percentage values. Online website (https://wpcalc.com/en/equilibrium-hardy-weinberg/) was used to evaluate the Hardy-Weinberg equilibrium (HWE) and the Chi square test was performed for determining the genotype frequencies in all groups. The statistical significance was considered at $p<0.05$. The analysis of variance (ANOVA) was performed to analyze the lipid profile parameters.

Results

164 males with CHD, with and without T2DM, participated in the study, with a mean age of 55.5 years, and 53 healthy individuals with a mean age 46.5 years were considered as a control group. Clinical characteristics at the time of diagnosis are listed in Table 1. All 217 individuals were examined successfully for genotype analysis in this work. The genotype frequencies of all groups were confirmed by HWE test, as I/I, I/D and D/D polymorphism in controls were 54.7%, 36% and 9.5% respectively and along with CHD, in T2DM patients, were 52%, 16.3% and 31.4% respectively, while CHD without T2DM were 84.6, 2.56, and 12.8, respectively, as shown in Table 2. No statistical significance was observed in allele frequency between control and CHD patients' groups. The allele frequencies for both groups are listed in Table 3. The frequency of I allele appeared higher than D allele in both groups. In addition, an association between CHD with T2DM and D/D genotype was observed; OR =4.3932; $p<0.0048$ as shown in Table 4. Furthermore, an association between CHD without T2DM and I/D genotype was shown; OR= 0.046; $p<0.0001$ as presented in Table 5.
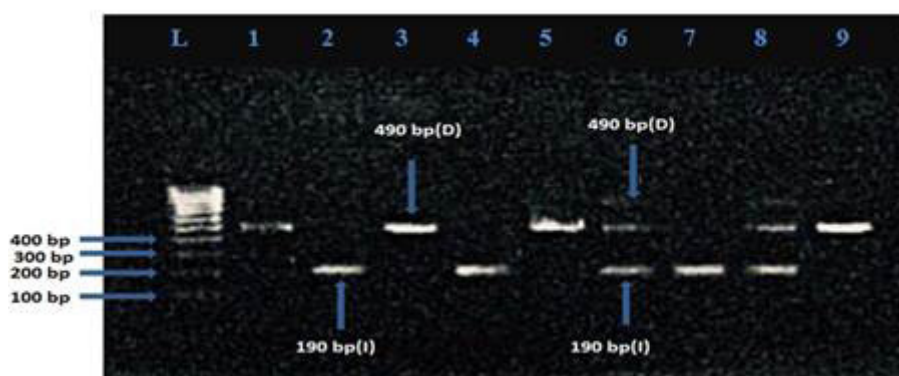

Figure 1: Genotypes of the ACE polymorphism (I/I, I/D and D/D)(rs4646994).

Table 1: The characteristics of CHD patients and control group participated in the study.

	I/I homozygous	I/D heterozygous	D/D homozygous
Control			
Male (N)	53	29	19	5
Age (year)	47	43	49	59
Cholesterol mg/dL	143±30.5	134.99±33.7	154.1±22.8	149.37±25.67
TG mg/dL	77.6±16.9	78.15±17	77.889±16.75	73.3±220.3
HDL mg/dL	38±3	38.4±12.3	37.8±10.1	39.7±16.65
LDL mg/dL	92±3174	9.41±34.11	88.89±31.2	101.5±20.5
VLDL mg/dL	15.52±3.4	15.63±33.4	15.6±3.35	14.67±4.1
CHD with T2DM			
Male (N)	86	45	14	27
Age (year)	57	54	69	66
Cholesterol (mg/dL)	176.6±44.3	188.2±45.9	148.7±33.6	171.8±40.1
TG (mg/dL)	187.26±12	201.5±91.8	125.4±19.4	195.6±83.6
HDL (mg/dL)	38.4±12.2	37.47±12.2	46.6±9.7	35.9±12.1
LDL (mg/dL)	135.2±33.84	137.88±33	141±37.6	127.6±33.16
VLDL (mg/dL)	31.4±11	34±13	25.47±5.7	30.2±7.8
CHD without T2DM			
Male (N)	78	66	2	10
Age (year)	54	55	63	47
Cholesterol (mg/dL)	162.4±24.5	165.45±24.2	144.5±12.1	145.9±21.1
TG (mg/dL)	162.4±72.4	174±72.7	99.5±1.4	98.8±17.2
HDL (mg/dL)	34.7±6.2	34.9±6.22	31.8±4.3	34.2±6.5
LDL (mg/dL)	141.6±49.2	148±48.7	93±29.2	109.8±39.4
VLDL (mg/dL)	34.3±14.5	36.8±14.31	21.4±2	20.5±3.7

Furthermore, the biochemical parameters were assessed in the control group, in addition to both groups of CHD patients, with and without T2DM, as demonstrated in table 6.

Discussion

Coronary heart disease is the most common polygenic disease that includes complex interactions between genes and several biochemical risk factors, making it the leading cause of death in many countries [15]. RAS has been recognized as a significant pathway for regulation of blood pressure, in addition to kidney function [16]. Therefore, the main aim of this study was to investigate the association between the ACE gene polymorphism and CHD in Iraqi patients, with and without T2DM. According to the World Health Organization (WHO), CHD represents the first cause of death in Iraq with 18.5% of total deaths. An extensive study suggested that polymorphisms in the constituents of RAS are significant in the development and consequences of CHD, within T2DM populations [17, 18], in addition to their role in atherosclerotic disease and related vascular complications [19]. The variation of ACE I/D in the intron 16 was shown to be implicated in the risk of CHD, and the frequency of allele deletion (DD) has been found to be higher in CHD with T2DM patients, relative to those carry I/I or I/D alleles (OR=3.48, p<0.02;

Table 2: The Hardy-Weinberg equilibrium for the ACE (I/D) polymorphism (rs4646994) in CHD patients and control groups, according to the expected and observed values

rs4646994	I/I	I/D	D/D	Total (N)	HWE p-Value & X^2	
Control						
Observed	29 (54.7)	19 (36)	5 (9.5)	53	0.47	
Expected	28	21	4		0.51	
MAF	0.27					
CHD with T2DM						
Observed	45 (52)	14 (16.3)	27 (31.4)	86	0.0001	
Expected	31.4	41.1	13.4		37.41	
MAF	0.4					
CHD without T2DM						
Observed (%)	66 (84.6)	2 (2.56)	10 (12.8)	78	0.0001	
Expected	57.6	18.9	1.6		62.364	
MAF	0.14					
SNP exact test for Hardy-Weinberg equilibrium (n=217)						
	N I/I	**N I/D**	**N D/D**	**N I**	**N D**	**p-Value**
All Subjects	140	35	42	315	119	<0.0001

MAF: Minor allele frequency

Table 3: The comparison of the ACE I/D allele frequencies among CHD patients and control groups

Allele	Control N (%) Total N=53	CHD Patients N (%) Total N=164	p-value
I	77 (72.6)	238 (72.5)	0.9871
D	29 (27.4)	90 (27.4)	
Total	106 (100)	328 (100)	

OR=4.39, p<0.0048, respectively). No statistical significance was observed for those who have CHD without T2DM (OR=0.878, p<0.8; OR=1.4, p value<0.55, respectively). The ACE allele frequencies in the healthy subjects were 72.6% for the I allele and 27.4% for the D allele, while the frequencies in the CHD group were 72.5% and 27.4% for the I and D alleles, respectively. Our results are consistent with other studies related to polymorphism at the same position [20, 21], whereas, Turkish and Gaza studies are not in line with our results [18, 22]. The main reasons are differences in both cases and healthy groups' selection criteria, such as age, BMI, hypertension and others, in addition to the risk of heritable factors in the investigated samples, bewildered by the little

information of parameters used. The usual factors like diabetes, obesity, and dyslipidemia have been reported in relation to the ACE gene in many issues [23]. Both, metabolic process and genetic factors, are appearing to be linked to severity, and susceptibility to, CHD [17]. In low- and middle-income countries, CHD deaths accounted for 80% of all deaths [24].

In general, the increase in age is associated with abnormalities in many arteries as there are a number of age-dependent changes in the structural components of the artery, which will lead to an increase in the number of elderly patients with heart disease [25]. Our results are in alignment with previous studies that reported on lipid metabolism disorders, suggesting an

Table 4: The association between the ACE (I/D) genotypes and the risk of CHD with T2DM

Genotype	Control N= 53	CHD with T2DM N= 86	OR (95% CI)	p value
Co-dominant				
II	29	45	1.00	
ID	19	14	0.4749 0.2064 to 1.0926	0.0798
DD	5	27	3.4800 1.2029–0.0675	0.0214
Dominant				
ID+DD	24	41	1.1009 0.5540–2.1878	0.7838
Recessive				
II+ID	48	59	1.00	
DD	5	27	4.3932 1.57231–2.2749	0.0048
Over dominant				
II+DD	34	72	1.00	
ID	19	14	0.3480 0.1561–0.7758	0.0099

Table 5: The association between the ACE I/D genotypes and risk of CHD without T2DM

Genotype	Control N=53	CHD without T2DM N= 78	OR (95% CI)	P value
Co-dominant				
II	29	66	1.00	
ID	19	2	0.046 0.0101–0.2117	0.0001
DD	5	10	0.8788 0.2758–2.8003	0.8270
Dominant				
ID+DD	24	12	0.2197 0.0969–0.4984	0.0003
Recessive				
II+ID	48	68	1.00	
DD	5	10	1.4118 0.4536–4.3938	0.5516
Over dominant				
II+DD	34	76	1.00	
ID	19	2	0.0471 0.0104–0.2136	0.0001

Table 6: Biochemical characteristics of control and CHD with and without T2DM

Parameters	Mean±SE		Group Comparison	P-Value
HDL (mg/dL)	Control		CHD with T2DM	0.928
	38.3±1.62		CHD without T2DM	.055
	CHD with T2DM			
	38.5±1.3		CHD without T2DM	.023
	CHD without T2DM			
	34.75±0.7			
LDL (mg/dL)	Control		CHD with T2DM	.000
	92±4.36		CHD without T2DM	.000
	CHD with T2DM			
	135±3.65		CHD without T2DM	.300
	CHD without T2DM			
	141.6±5.57			
VLDL (mg/dL)	Control		CHD with T2DM	.000
	15.52±0.46		CHD without T2DM	.000
	CHD with T2DM			
	31.4±1.2		CHD without T2DM	.090
	CHD without T2DM			
	34.38±1.64			
TG (mg/dL)	Control		CHD with T2DM	.000
	77.6±2.32		CHD without T2DM	.000
	CHD with T2DM			
	187.25±9.2		CHD without T2DM	.023
	CHD without T2DM			
	162.4±8.2			
CH (mg/dL)	Control		CHD with T2DM	0.000
	143.2±4.2		CHD without T2DM	0.002
	CHD with T2DM			
	176.6±4.7		CHD without T2DM	0.01
	CHD without T2DM 162.4±2.8		CHD without T2DM	

Bold values are statistically significant.

essential role in atherosclerosis progress in CHD patients. They appeared elevated in all lipid constituents except HDL that came with low levels. In most countries, dyslipidemia is considered important as a dynamic risk factor for atherosclerosis, and confirmed for CHD [26] where lipid profiles' disturbances in plasma lead to an increase susceptibility, causing CHD [27]. A number of mechanisms are suggested that clarify the strong consequences of lipid profile on the CHD, depending on the physiological role of lipids [28]. HDL carries about 20% of the total plasma cholesterol, transporting excess cholesterol from the arterial wall's foam macrophages to

the liver [29], causing the outflow of cholesterol from peripheral cells. A potent association exists between low levels of HDL and the risk for atherosclerosis. On the other hand, LDL, as a major atherogenic lipoprotein, supports cholesterol accumulation in the vessel wall, causing a hindrance of blood flow.

Our work is relatively limited due to the small sample size. We strongly suggest further investigations on a larger number of cases that may validate the significance of ACE I/D polymorphism in the pathobiology of CHD.

Conclusions

So, we have concluded that D/D genotype of the ACE polymorphism (rs4646994) is associated with more than 4-fold higher predisposition to CHD. However, the dyslipidemia was also implicated in the severity of CHD.

Acknowledgement

Conflicts of interest

The authors declare no conflict of interest.

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