Relationship of angiotensin converting enzyme (I/D) polymorphism (rs4646994) and coronary heart disease among a male Iraqi population with type 2 diabetes mellitus

Raghda N. Hemeed¹ · Fadhil J. Al-Tu'ma¹ · Dhafer A. F. Al-Koofee² · Ahmed H. Al-Mayali³

Received: 15 May 2020 / Accepted: 7 September 2020 / Published online: 30 September 2020
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Abstract

Background Insertion deletion (I/D) polymorphism (rs4646994) in the angiotensin-converting enzyme (ACE) has a substantial effect on coronary heart disease (CHD). 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.

Objective The objective of this study was to investigate the association between the *ACE* gene polymorphism and CHD among male Iraqi patients with and without type 2 diabetes mellitus (T2DM).

Methods A case-control study of total 217 male subjects participated in this study, divided into three groups; Group 1 including 86 CHD patients with T2DM, group 2 including 78 CHD patients without T2DM, and group 3 including 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.

Results The II allele was significantly more frequent in CHD patients without T2DM compared to the control population, but not from those patients with T2DM ($p < 0.05$). Nonetheless, the ID allele was significantly more frequent in each of CHD with T2DM and control populations compared to the CHD without T2DM. The DD allele was significantly more frequent in CHD patients with T2DM compared to each of CHD patients without T2DM and control populations ($p < 0.05$).

Conclusion We conclude that the D/D and I/D genotypes are implicated as risk factors for development of CHD with T2DM, but not CHD without T2DM among the male Iraqi population. However, larger sample sizes are needed to monitor the CHD patients and to validate this study.

Keywords Ischemic heart disease · Coronary heart disease · Angiotensin converting enzyme · Type 2 Diabetes Mellitus

Introduction

The coronary heart disease (CHD) is associated with fat accumulation at the end of the atrium, reducing the arteries' cavity, retarded blood flow, and consequently ischemic heart failure. Because of the high morbidity rate, the CHD has become one

of the most life-threatening cardiovascular diseases with more than one-third of death cases among those with ≥ 35 years [1–4]. It may affect individuals at all ages, though being significantly more common in the elderly with higher rate among males. Noticeably, common risk factors include smoking, familial history, hypertension, overweightness, diabetes, alcohol consumption, lack of exercise, stress, and hyperlipidemia [5]. Symptoms of Ischemic Heart Disease (IHD) or CHD include angina (severe chest pain during exertion) and heart palpitation due to decrease or lack of exercise. The CHD is diagnosed using 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, 7]. Partial or complete blocked arteries cause angina or heart attack, respectively, due to the gradual death of heart cells [8, 9]. Each year, approximately 790,000 adults suffer from myocardial infarction (MI), and

✉ Dhafer A. F. Al-Koofee
dhafera.faisal@uokufa.edu.iq

¹ Department of Biochemistry, College of Medicine, University of Kerbala, Kerbala, Iraq

² Department of Clinical Laboratory Science, Faculty of Pharmacy, University of Kufa, Najaf, Iraq

³ Department of Internal Medicine, College of Medicine, University of Kerbala, Kerbala, Iraq

210,000 of them suffer from frequent heart attacks [10]. Somatic angiotensin-I converting enzyme (sACE) plays an important role in the blood pressure regulation and electrolyte fluid balance. It is a zinc protease that cleaves angiotensin-I (AngI), bradykinin, and a large group of other signal peptides [11]. 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 I/D genotype is significantly associated with cardiovascular disease (CVD) development [13, 14]. Our objective was assessment the relationship of angiotensin converting enzyme (I/D) polymorphism (rs4646994) and male Iraqi CHD patients with T2DM compared to the healthy control population.

Materials & methods

This study was ethically approved by Department of Biochemistry, College of Medicine, University of Kerbala, Iraq. As a case-control survey, herein 217 male subjects were classified into three groups. The group I composed of 86 patients with IHD/CHD and Type 2 diabetes mellitus (T2DM); the group II included 78 patients, having IHD without T2DM; and the group III included 53 healthy individuals without any diseases (control group). Patients' inclusion criteria included last coronary event more than 6 months prior to enrollment. Samples were collected from Al-Hussein Medical City, Kerbala. The exclusion criteria for T2DM included (1) Female; (2) Type 1 diabetes mellitus; (3) Patients treated with catheterization; (4) Children and (5) severe diseases or a life expectancy of less than 5 years. The inclusion criteria were as follows: (1) The selected patients diagnosed with one of the following diseases, T2DM, hypertension, myocardial infarction (MI) and unstable angina; (2) Adult male patients; (3) Before performing catheterization; (4) those with smoking and with familial history of T2DM.

Blood was taken (5 ml) from all participants and divided into three parts: One milliliter for molecular analysis (DNA extraction) in a tube, 1 mL for HbA1c analysis both with EDTA and 3 mL for biochemical analysis.

Total DNA was extracted using the genomic DNA extraction Kit (Geneaid Biotech Ltd., UK), according to the manufacturers' protocol. Concentration and purity of isolated DNA were measured by a BioDrop (UK). The existence of the I and D alleles of the ACE was detected by PCR, according to the assay 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 min, and 35 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 40 s, and finally one cycle at 72°C for 5 min. Lastly, 7 µl of amplicon was separated by electrophoresis on a 2% agarose gel, containing safe stain and finally visualized by a transilluminator. The product of I allele was a 490 bp band, while the D allele was a 190 bp band of DNA, and I/D genotype was confirmed by amplification of two products with 490 bp and 190 bp sizes.

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. As well as 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

One hundred-sixty four 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 % and along with CHD in T2DM patients were 52, 16.3 and 31.4 %, 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 and T2DM or D/D genotype was observed (OR = 4.3932; $p < 0.0048$). Furthermore, an association between CHD without T2DM and I/D genotype was shown (OR = 0.046; $p < 0.0001$) (table3).

According to the Table 3, the II allele was significantly more frequent in CHD patients without T2DM compared to the control population, but not from those patients with

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

Characteristics	mean $\pm$ SD	I/I homozygous	I/D heterozygous	D/D homozygous
Control				
Male (N)	53	29	19	5
Age (year)	46.5	43	49	59
Cholesterol mg/dL	143 $\pm$ 30.5	134.99 $\pm$ 33.7	154.1 $\pm$ 22.8	149.37 $\pm$ 25.67
TG mg/dL	77.6 $\pm$ 16.9	78.15 $\pm$ 17	77.889 $\pm$ 16.75	73.3 $\pm$ 220.3
HDL mg/dL	38 $\pm$ 3	38.4 $\pm$ 12.3	37.8 $\pm$ 10.1	39.7 $\pm$ 16.65
LDL mg/dL	92 $\pm$ 3174	9.41 $\pm$ 34.11	88.89 $\pm$ 31.2	101.5 $\pm$ 20.5
VLDL mg/dL	15.52 $\pm$ 3.4	15.63 $\pm$ 33.4	15.6 $\pm$ 3.35	14.67 $\pm$ 4.1
CHD with T2DM				
Male (N)	86	45	14	27
Age (year)	55.5	54	69	66
Cholesterol (mg/dL)	176.6 $\pm$ 44.3	188.2 $\pm$ 45.9	148.7 $\pm$ 33.6	171.8 $\pm$ 40.1
TG (mg/dL)	187.26 $\pm$ 12	201.5 $\pm$ 91.8	125.4 $\pm$ 19.4	195.6 $\pm$ 83.6
HDL (mg/dL)	38.4 $\pm$ 12.2	37.47 $\pm$ 12.2	46.6 $\pm$ 9.7	35.9 $\pm$ 12.1
LDL (mg/dL)	135.2 $\pm$ 33.84	137.88 $\pm$ 33	141 $\pm$ 37.6	127.6 $\pm$ 33.16
VLDL (mg/dL)	31.4 $\pm$ 11	34 $\pm$ 13	25.47 $\pm$ 5.7	30.2 $\pm$ 7.8
CHD without T2DM				
Male (N)	78	66	2	10
Age (year)	55.5	55	63	47
Cholesterol (mg/dL)	162.4 $\pm$ 24.5	165.45 $\pm$ 24.2	144.5 $\pm$ 12.1	145.9 $\pm$ 21.1
TG (mg/dL)	162.4 $\pm$ 72.4	174 $\pm$ 72.7	99.5 $\pm$ 1.4	98.8 $\pm$ 17.2
HDL (mg/dL)	34.7 $\pm$ 6.2	34.9 $\pm$ 6.22	31.8 $\pm$ 4.3	34.2 $\pm$ 6.5
LDL (mg/dL)	141.6 $\pm$ 49.2	148 $\pm$ 48.7	93 $\pm$ 29.2	109.8 $\pm$ 39.4
VLDL (mg/dL)	34.3 $\pm$ 14.5	36.8 $\pm$ 14.31	21.4 $\pm$ 2	20.5 $\pm$ 3.7

T2DM. Nonetheless, the ID allele was significantly more frequent in each of CHD with T2DM and control populations compared to the CHD without T2DM. The DD allele was

significantly more frequent in CHD with T2DM patients compared to each of CHD without T2DM and control populations. The ID+DD genotype was significantly more frequent in

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 <i>p</i> Value & χ^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 (<i>n</i> = 217)					
	N I/I	N I/D	N D/D	N I	N D
All Subjects	140	35	42	315	119
					P Value
					<0.0001

MAF: Minor allele frequency

Table 3 The association between the *ACE* I/D genotypes and risk of CHD without T2DM using ANOVA test

Genotype	CHD without T2DM N = 78, N (%)	95% CI	CHD with T2DM N = 86, N (%)	95%CI	Control N = 53, N (%)	p value
II	66 (84.61)	0.01–0.21	45 (52.32)	0.11–1.12	29 (54.71)	p = 0.001
ID	2 (2.56)	0.22–2.80	14 (16.27)	0.21–1.1	19 (35.84)	p < 0.0001
DD	10 (12.82)	0.09–0.49	27 (31.39)	0.33–1.00	5 (9.43)	p < 0.0001
ID+DD	12 (15.38)	0.11–0.74	41 (47.67)	1.4–8.21	24 (45.28)	p = 0.001
II + ID	68 (87.17)	0.49–4.46	59 (68.60)	0.43–2.01	48 (90.56)	p = 0.101
II + DD	76 (97.43)	0.01–0.14	42 (48.83)	1.57–12.28	34 (64.15)	p = 0.001

CHD with T2DM and control groups compared to the CHD without T2DM population. However, the II + ID genotype was not significantly different neither between the CHD without T2DM and control nor the control and CHD with T2DM populations. Regarding the II + DD genotype, there was a significant difference between the CHD without T2DM and control populations.

Furthermore, regarding the biochemical parameters, the HDL ($p = 0.023$), LDL ($p = 0.0001$), VLDL ($p = 0.0001$), TG (0.0001) and CH (0.01) were significantly different between CHD patients with T2DM and those without T2DM.

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 accounting for 18.5%. 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 value < 0.02; OR=4.39, p value < 0.0048, respectively). No statistical significance was observed for those who have CHD without T2DM (OR=0.878, p value < 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 were consistent with other studies related to polymorphism at the same position [20, 21], whereas Turkish and Gaza studies were 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 percent of all mortalities [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 essential role in atherosclerosis progress in CHD patients. They appeared elevated in all lipid constituents except HDL that come 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 size collection. 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

We concluded that the D/D (rs4646994) and also I/D genotypes of the *ACE* were implicated as risk factors for development of CHD with T2DM, but not CHD without T2DM among the male Iraqi population. However, larger sample sizes are needed to monitor the CHD patients and to validate this study.

Acknowledgements We would like to thank all the staff of laboratory of Al-Hussein Medical City and the participants of both healthy and CHD groups for their cooperation.

Compliance with ethical standards

Competing interests The authors declare to have no competing interests.

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